Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Department of Plant Biology

Biological Control and Growth Promotion in *Solanum spp.*

Johannes Klint and Niklas Zeiner

Degree project • 15 credits
Agriculture Programme - Soil and Plant Sciences
Uppsala 2020

Biological Control and Growth Promotion in *Solanum spp.*

Johannes Klint and Niklas Zeiner

Supervisor:	Johan Meijer, Swedish University of Agricultural Sciences, Department of Plant Biology
Assistant supervisor:	Mikael Pell, Swedish University of Agricultural Sciences, Department of Molecular Sciences
Assistant supervisor:	Björn Andersson, Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology
Examiner:	Folke Sitbon, Swedish University of Agricultural Sciences, Department of Plant Biology

Credits:	15 credits
Level:	First cycle, G2E
Course title:	Independent Project in Biology
Course code:	EX0894
Programme/education:	Agriculture Programme - Soil and Plant Sciences
Course coordinating department:	Department of Plant Biology
Place of publication:	Uppsala
Year of publication:	2020
Online publication:	https://stud.epsilon.slu.se
Keywords:	Plant Growth Promoting Rhizobacteria, Biological Control, *Solanum tuberosum*, *Phytophthora infestans*, *Alternaria solani*

Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology

Abstract

Potato (*Solanum tuberosum*) is Sweden's most treated crop in terms of chemical disease control, primarily to prevent potato late blight caused by *Phytophthora infestans*. Another important disease is potato early blight caused by *Alternaria solani*. In this thesis biological disease control was explored as a supplement or an alternative to chemical disease control. The idea was to isolate, identify and apply bacteria on potato plants and examine their effects on growth and disease control.

Two bacterial strains were isolated from the potato relative bittersweet nightshade (*Solanum dulcamare*), assuming that these bacteria would also colonize potato roots. Screening for suitable bacteria used tissue prints and biofilm forming capability.

Identification of the bacterial species was based on partial gene sequencing of the 16S rRNA gene and several housekeeping genes as well as biotests.

Initial gene sequencing results did not completely match any bacteria in the NCBI database, but indicated relatedness to the genus *Stenotrophomonas*. Since certain *Stenotrophomonas* strains are opportunistic human pathogens, time was re-allocated to further investigate the identity before further applied work with the bacteria.

Many *Stenotrophomonas* strains are plant-associated and candidates for growth promotion and biological control according to literature. The growth promoting effect of the two bacteria was weak but partly statistically significant in greenhouse tests. The *in vitro* experiments with disease control of *P. infestans* and *A. solani* were difficult to evaluate. Pathogen inoculation of potato leaves previously treated with the bacterial strains, indicated a control effect in certain bacteria/pathogen-combinations. n tubers however, pathogen inoculation resulted in no disease symptoms neither in the control group nor in the group pre-treated with the bacterial strains. These experiments should be regarded as pilot studies and given published studies, additional experiments should be conducted with our isolates.

Growth promotion and biological control based on beneficial plant-microbe-interaction will play an important role in future crop production and pest management, either as a complement or as a substitute to chemicals. Challenges are to increase efficacy of these treatments in complex biological environments and assure successful transfer of *in vitro* effects to field conditions.

Keywords: Plant Growth Promoting Rhizobacteria, Biological Control, Solanum tuberosum, Phytophthora infestans, Alternaria solani

Sammanfattning

Potatis (*Solanum tuberosum*) är den mest besprutade grödan i Sverige. Det är i synnerhet potatisbladmögel (*Phytophthora infestans*) som orsakar brunröta som bekämpas. Även torrfläcksjuka, som orsakas av *Alternaria solani*, är ett stort problem. I vårt arbete undersökte vi möjligheterna till biologisk kontroll som ett komplement eller alternativ till kemisk bekämpning. Syftet var att isolera, identifiera och applicera bakterier på potatis samt undersöka effekter på tillväxt och biologisk kontroll.

Isolering av två nya bakteriestammarna gjordes ifrån rötterna av potatissläktingen besksöta (*Solanum dulcamara*) då förmågan att kolonisera potatisrötterna bedömdes som goda. Lämpliga kolonier valdes utifrån visuella bedömningar av ”tissue prints” och förmågan att bilda biofilm.

Identifiering av bakterierna gjordes genom gensekvensering och analys av 16S rRNA genen och ett antal ”housekeeping” gener samt biotester.

Resultatet av gensekvenseringen av bakteriestammarna matchade inte fullständigt med någon bakterie i NCBIs databas, men tydde på att de tillhörde släktet *Stenotrophomonas*. Då vissa stammar av *Stenotrophomonas* är opportunistiska humanpatogener lades mycket tid på vidare identifiering av bakterierna för att bedöma riskprofilen innan fortsatt växtarbete.

Många *Stenotrophomonas* stammar är växtassocierade och kan ge tillväxtstimulering och biologisk kontroll enligt litteraturen. Våra resultat visade dock att inverkan på friska potatisplantors tillväxt var svag men delvis statistiskt signifikant i växthus. *In vitro* försök med *P. infestans* och *A. solani* var svåra att utvärdera. Bladförsöken gav en viss indikation om att en skyddande effekt kunde urskiljas i en grupp. Knölförsöket kunde dock inte utvärderas på grund av utebliven patogeninfektion. Försöken kan betraktas som pilotstudier och med tanke på tidigare undersökningar bör våra isolat undersökas vidare.

Tillväxtstimulering och biologisk kontroll baserad på gynnsamma växt-mikrob-interaktioner kommer att spela en viktig roll i framtidens produktion av jordbruksgrödor och växtskydd som komplement eller substitut för kemiska preparat. Den stora utmaningen är att öka verkningsgraden av biologiska preparat i komplexa odlingssystem och förbättra överföring av goda *in vitro* effekter till fält.

Nyckelord: Plant Growth Promoting Rhizobacteria, Biological Control, Solanum tuberosum, Phytophthora infestans, Alternaria solani

Preface

It began with working for Johan Meijer during the summer of 2018. He tested a novel strain of Bacillus (5113) on oilseed rape plants for its effect on root formation, growth stimulation and protection against abiotic stress. This opened our minds regarding the prospect of developing beneficial plant-microbe interactions and its use in the future of farming.

Our first intention was to test already identified and confirmed PGPRs and applying them on potatoes. Primarily we wanted to test them for their ability as biological control agents against oomycete and fungal pathogens. Instead of doing that, we choose a more offensive approach and decided to isolate novel bacteria on our own.

We planned to isolate bacteria from wild plant material and then study the effect of these bacteria on selected potato pathogens and plant growth. Our intention was also to identify the isolated bacteria and to learn more about some taxonomical methods (PCR, bioinformatics and biochemical tests).

Table of contents

1 Introduction

An estimation of the annual cost due to the oomycete *Phytophthora infestans,* the causative agent of Potato late blight, is 5 Billion USD (about 47.5 billion SEK) (Judelson and Blanco, 2005). This number was based on both the actual crop losses due to the disease, as well as the cost of the control measures. The expensive control measures are mainly due to the large quantities of fungicides used to manage *P. infestans.*

Potatoes are grown vegetatively and their high degree of heterozygosity (potatoes are autotetraploid) complicates breeding of new varieties with desirable qualities such as resistance towards potato late blight. In addition, *P. infestans* has the ability of sexual recombination, hence rapidly changing genotype, which makes it adaptable to changing circumstances such as climate, plant resistance as well as fungicides aimed to defeat it (Dixelius, 2012).

Potato is generally a pest sensitive crop and its cultivation requires the use of pesticides (e.g fungicides). In Sweden, potato is only grown on 1 % of the farmed land area, but uses on average 40 % of the total fungicides. On average every potato crop is treated 7.5 times before harvest but the number of treatments varies between 3-12 per season during conventional farming practices (SCB, 2006).

The intensive use of fungicides creates environmental issues but also raises economic concerns. Further, intensive use of fungicides speeds up pest resistance development making chemical control useless.

In order to secure future yields within the agricultural sector, there will always be a need for plant protection agents, both chemical and biological. One of the environmental goals, Non-Toxic Environment ("giftfri miljö") is about minimising the residues of synthetic chemical substances in our food and environment. Combined with development of pathogen resistance towards active substances in pesticides and the environmental impact of synthetic fertilizers, this makes the use of novel strains of plant associated rhizobacteria an interesting case for application within the agricultural sector.

This somewhat problematic scenario caught our interest in searching for an alternative or complementary method of pest control, namely biological control.

Our aim was to try to isolate and identify a plant associated bacterium from wild material, propagate it, and then apply this in a given concentration under controlled settings, to study the potential effect on plant growth and disease protection towards

two common pathogens associated with potatoes (*P. infestans* and *Alternaria solani*).

1.1 Rhizosphere bacteria

The narrow zone surrounding plant roots is referred to as the rhizosphere. This is considered one of the most complex ecosystems on earth as it contains groups of microorganisms that can be harmful, neutral or beneficial in relation to the plant (Bardgett and van der Putten 2014.)

Through the process of photosynthesis, plants have developed the ability to fix atmospheric CO_2 into reduced carbon, which makes up the building blocks for organic compounds. Studies indicates that up to 40% of these compounds (fatty acids, nucleotides, amino acids, sterols, vitamins, sugars, etc.) become root exudates and released into the rhizosphere. This exudation is considered as a strategy to feed and attract heterotrophic bacteria and fungi that are likely to be beneficial for the plant (Hardoim et al, 2008, Lugtenberg and Kamilova, 2009, Compant et al, 2010). The rhizosphere is much richer in bacteria than the surrounding bulk soil (Hiltner, 1904). The population density of just the root surface have been estimated to about 10^5-10^7 CFU/g fresh weight, and 10^7-10^9 CFU/g of soil within the rhizosphere. Diversity wise, more than 4 000 microbial species are estimated to be present within 1 gram of soil (Hardoim et al, 2008, Lugtenberg and Kamilova, 2009, Compant et al, 2010).

The significantly elevated number of microorganisms surrounding the near surface of the roots is commonly known as the rhizosphere effect. More specifically the root exudates are regarded as the primary force that regulates microbial activity and diversity around the root surface as well as the entire structure of the microbial community (Philippot et al, 2013; Jones et al, 2009).

The composition of root exudates also implies that plants might be able to modulate the root microbiome to their benefit, for instance via protection against pathogens or by assisting the plants to acquire nutrients (Gkarmiri, 2018).

1.2 PGPR properties

Plant associated bacteria can be described as epiphytic or endophytic with regard to the position of their ecological niche in relation to the plant (Figure 1). Epiphytic bacteria live outside the root in the rhizosphere or rhizoplane, while endophytic bacteria have their life cycle partly or entirely in the intra- and intercellular spaces of plant tissues (dos Santos et al, 2018).

Microorganisms can also be described based on their actual impact on plant growth. According to Whipps (2001) the interaction between a bacterium and a

plant can be categorized as positive, neutral or negative. When the interaction is neutral the bacteria are classified as commensal; when negative, they are regarded as pathogens or parasitic; and if positive, they are called mutualists. In all these interactions, the microbe benefit through assimilation of nutrients from the plant (Hentschel et al, 2000; Hirsch, 2004).

If the interaction is mutualistic and significantly improves plant growth these bacteria are usually referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper et al, 1989). Criteria that must be met for a bacterium to become a PGPR are good rhizosphere competence, efficient plant colonization and growth promotion. PGPR can colonize plants as epiphytes or endophytes (Gray and Smith, 2005). Current estimates suggest that only 1 to 2% of the bacteria in the rhizosphere promote growth stimulation of a plant (Antoun and Kloepper, 2001). The predominant genera of PGPR are *Bacillus* and *Pseudomonas* (Podile and Kishore, 2006).

Stimulation of plant growth can be direct or indirect (Figure 2). Direct stimulation can be due to for instance bacterial synthesis of phytohormones such as auxin (IAA) or by providing the plant with limiting nutrients such as fixing nitrogen or solubilisation of minerals in the soil. Indirect effects can be due to decreasing or stopping the effects of pathogenic or parasitic organisms. This in turn can be achieved through direct antagonism such as chemical warfare (antibiosis), e.g. production of fungicides, antibiotics, enzymes that lyse fungal cell walls or secondary metabolites such as hydrogen cyanide that inhibit the growth of pathogens. Indirect ways of PGPR to protect against pathogens are by competition (if they share a similar ecological niche around the plant) or by making nutrients inaccessible to the pathogen. A well-documented example on the latter is the production of siderophores that chelates iron, thereby preventing its utilization by pathogens. The last well known indirect mechanism is by upregulation of the plants own defence through priming of induced systemic resistance (ISR)(Glick, 1995; Singh, 2013).

PGPRs can be assigned to different categories based on their modes of action, e.g. biostimulants, biofertilizers or biopesticides. In the ideal scenario, a PGPR belongs to several such categories (Lugtenberg and Kamilova, 2009). PGPRs have in certain cases become commercially viable in agricultural applications since there is an interest for a reduction of chemical use and a rising demand for ecological alternatives.

The process of root colonization by PGPR is not dependent on a single variable and is thus not a uniform process. Factors such as root exudates, conditions of the soil (pH, texture, moisture, microbial composition etc.), bacteria-bacteria communication, and plant-bacteria communication influence the process of colonization (Hardoim et al, 2008, Lugtenberg and Kamilova, 2009, Compant et al, 2010).

The colonization process often starts with the bacteria being able to recognize specific compounds or hydrated polysaccharides exudated from plant roots, that thereby provide a trigger of communication. This initiates the actual process of bacterial colonisation of the rhizosphere, or rhizoplane. Through these compounds

and secondary metabolites, plants are believed to communicate with both beneficial as well as harmful rhizobacteria, fungi and other soil organisms (Hardoim et al, 2008, Compant et al, 2010).

It is experimentally difficult to study solely the effect of a specific PGPR in soil. The PGPR can change the structure of the entire microbial community in the rhizosphere. Processes like quorum sensing/regulation (density dependent activities) will also influence the outcome. Depending on the soil and microbial community that is used the result of a treatment with a specific PGPR can vary (Lugtenberg and Kamilova, 2009). Thus, specific bacteria can show plant growth promotion during *in vitro* screening, but may face problems to sustain a critical population density under field conditions.

More specifically, the phenomenon of quorum sensing denotes communication between kin bacteria through signal molecules, which helps them organise their behaviour. The coordination is governed in relation to their population density and involves processes as root colonization. Such bacteria can sense the concentration of signal molecules and regulate the transcription of genes related to the colonization process accordingly. Examples of genes that are usually upregulated are genes related to the production of antibiotics and biofilm formation (Lugtenberg & Kamilova, 2009; Compant et al, 2010).

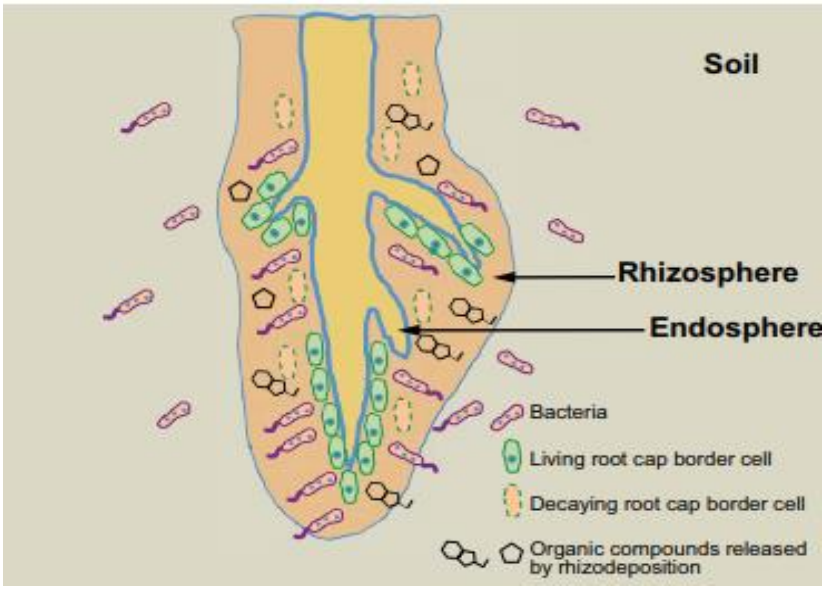

Figure 1. Different niches in the root-soil interface. Specifically root-associated bacteria that communicates traits associated with root colonization. (Adapted from Bulgarelli et al, 2013)

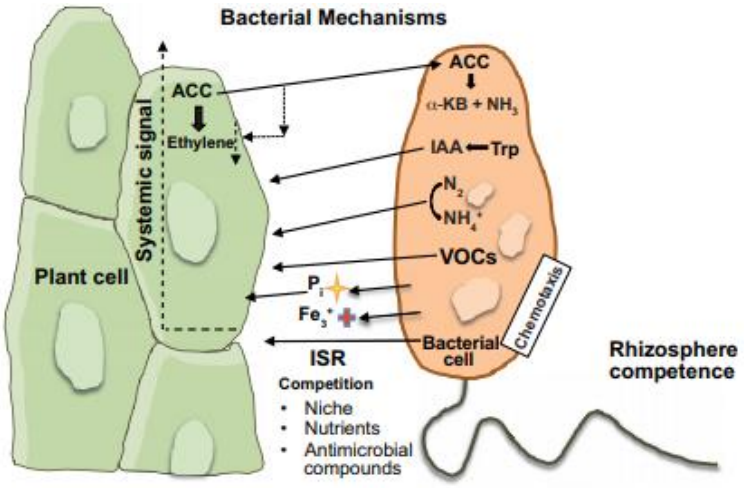

Figure 2: PGPR and mechanisms described to promote plant growth. Production of phytohormones, nitrogen fixation, solubilization of phosphorus, siderophore production, induced systemic resistance (ISR) etc. (Adapted from Bulgarelli et al, 2013)

1.3 Biological control

The term Biological Control Agent (BCA) is often used in plant pathology and entomology. It applies to the use of microbial antagonism against plant-associated pathogens. Microbes to be commercialized to protect crops against pathogens, are referred to as biopesticides.

In more general terms, microbial antagonism can be exhibited through various modes of action that are not mutually exclusive. It can involve direct interactions between the biocontrol agent and the pathogen, as well indirect interactions where the plant responds to the presence of the antagonist. The direct interaction involves modes of action known as antibiosis (secretion of secondary metabolites harmful to the pathogen), competition (nutrients and space) as well as parasitism (biocontrol agent infects the pathogen). The indirect antagonism can be the activation of Induced Systemic Resistance (ISR) through priming, or by direct growth promotion that increases the plants general health (biocontrol agents increase availability of nutrients or/and production of phytohormones). These modes of actions can often be exhibited sequentially or simultaneously by a single biocontrol agent (Kamal et al, 2015).

1.3.1 Competition

Competition can occur at different levels between an antagonist and a pathogen, both with regards to space of certain infection sites or ecological niches on the roots

and seeds. A well understood mechanism of biocontrol is the competition between microorganisms for limiting nutrients in the rhizosphere. This competition can occur for macronutrients like carbon, nitrogen or phosphorus, but also micronutrients such as iron. In soils where iron is a limiting nutrient, the production of iron-chelating siderophores has been shown as a mechanism of biocontrol performed by several species of bacteria. Fluorescent *Pseudomonas spp*. are recognized for their ability to produce a wide range of siderophores such as pyoverdines and pseudobactins. This is regarded as a strategy for the bacteria to outcompete pathogenic fungi and other deleterious microorganisms by sequestering the limited amount of iron in the rhizosphere, therefore making it unavailable for them to use (Pankhurst and Lynch, 2005).

1.3.2 Antibiosis

Antibiosis is basically the secretion of low molecular weight antibiotics (among other secondary metabolites) which can either kill or inhibit the growth of other microorganisms. In agriculture, one would like to utilize microorganisms beneficial to the plant that can produce such compounds, thereby working as biocontrol agents towards pathogenic or deleterious microorganisms (Pankhurst and Lynch, 2005).

A well-known example is antibiosis of a *Pseudomonas fluorescens* strain that demonstrates strong antagonism against the fungal pathogen *Gaeumannomyces graminis* that causes take-all of wheat. An identified mechanism behind this antagonism is the production of phenazine-carboxylic acid. *P. fluorescens* also produces the antibiotic 2,4-diacetylphloroglucinol that have been proven effective against damping-off of sugar beet (Pankhurst and Lynch, 2005).

1.3.3 Induced systemic resistance (ISR)

ISR is a mechanism induced by non-pathogenic rhizobacteria that stimulates the plant´s own defence system towards pathogens (Pieterse et al, 1998). The ISR defence differs in some aspects from Systemic Acquired Resistance (SAR). SAR is induced by pathogenic organisms or non-biotic agents and usually characterized by an increased level of endogenously produced salicylic acid as well as pathogenesis-related (PR) proteins. ISR on the other hand uses an upregulation of jasmonic acid and increased ethylene production to activate its defence systems and seems to induce only a few PR proteins (Hammerschmidt, 1999; van Wees et al, 2000; Pieterse, 2002). ISR is more recently discovered and with not well understood mechanisms. Examples of common PR-proteins are glucanases and chitinases. ISR might provide biocontrol toward soil-borne pathogens as well as foliar pathogens. Although ISR is difficult to study and quantify, the actual importance of ISR can be great because it operates as an indirect mechanism, and because biocontrol agents often work in multivariable ways (Pankhurst and Lynch, 2005).

Biological control agents, when commercialized, can be registered based on the mode of protection. If classified as biostimulants the product should not act *per se,* but stimulate the plant´s own resources, e.g. increase the general defense system and strength of the plant through ISR or the production of phytohormones. They can also increase pathogen resistance through the sequestering and provision of nutrients; hence this indirectly increases the overall health of a plant. The most obvious products are registered as bioprotectants, supposed to be direct suppressors of pathogens through antibiosis and spatial occupation. Common genera of bacteria found associated with these abilities are *Bacillus spp.*, *Pseudomonas spp.*, *Burkholderia spp.*, *Streptomyces spp.*, *Paenibacillus spp.* and *Agrobacterium spp*. (Kamal et al, 2015) but there are many other examples of bacteria connected with disease and pathogen resistance (Table 1).

PGPR	**Disease resistance**
Bacillus pumilus, *Kluyvera cryocrescens*, *B. amyloliquefaciens* and *B. subtilus*	Cucumber Mosaic Cucumovirus (CMV) of tomato (*Lycopersicon esculentum*)
B. amyloliquefaciens, *B. subtilis* and *B. pumilus*	Tomato mottle virus
B. pumilus	Bacterial wilt disease in cucumber (*Cucumis sativus*), Blue mold disease of tobacco (*Nicotiana*)
Pseudomonas fluorescens	Sheath blight disease and leaf folder insect in rice (*Oryza sativa*), Reduce the Banana Bunchy Top Virus (BBTV) incidence, Saline resistance in groundnut (*Arachis hypogea*)
B . subtilis and B. pumilus	Downy mildew in pearl millet (*Pennisetum glaucum*)
B. subtilis	CMV in cucumber
B. cereus	Foliar diseases of tomato
Bacillus spp.	Blight of bell pepper (*Capsicum annuum*), Blight of squash
Burkholderia	Maize (*Zea mays*) rot
B. subtilis	Soil borne pathogen of cucumber and pepper (*Piper)*
Bacillus sp. and *Azospirillum*	Rice blast
Fluorescent *Pseudomonas* spp.	Rice sheath rot (*Sarocladium oryzae*)

Table 1. Examples of PGPR strains and their disease/pathogen targets (Singh, 2013)

An example of biopesticides within the agricultural sector in Sweden is the use of *Pseudomonas chlororaphis*, which is used against seed-borne diseases and have been commercialized as three different biopesticide formulations by the company BioAgri (Lantmännen)(https://www.bioagri.se/).

Pseudomonas spp. are known as competent root colonizers as well as having the ability to produce antifungal substances such as proteases, hydrogen cyanide, chitinases and phenazine-1-carboxamid (PCN). The ability to spatially compete against pathogens combined with the antibiosis ability makes them attractive biocontrol agents (Bloemberg et al, 2001). So, even though the same bacteria may be used, the commercial products can differ with respect to their actual branding as biopesticides. This is based on differences in application mode, formulation and proven effect against certain pathogens. BioAgris specific formulation in barley and oats is called Cedomon ®. The product against seed-borne diseases in pea and carrot is referred to as Cedress® while in wheat the name of the product is Cerall ® (https://www.bioagri.se/).

2 Method/Pilot experiments

In order to be able to separate frequently occurring "common" soil bacteria from bacteria with potential novelty (regarding plant association), we began with a screening process of soil. We performed "soil prints" by applying different soil samples on Luria broth (LB) nutrient agar. Thereby we had some visual references about what kind of bacteria that appeared after applying soil on LB agar. One striking observation was that different forms of actinomycetes seemed to be predominant in the soil samples (Figure 3).

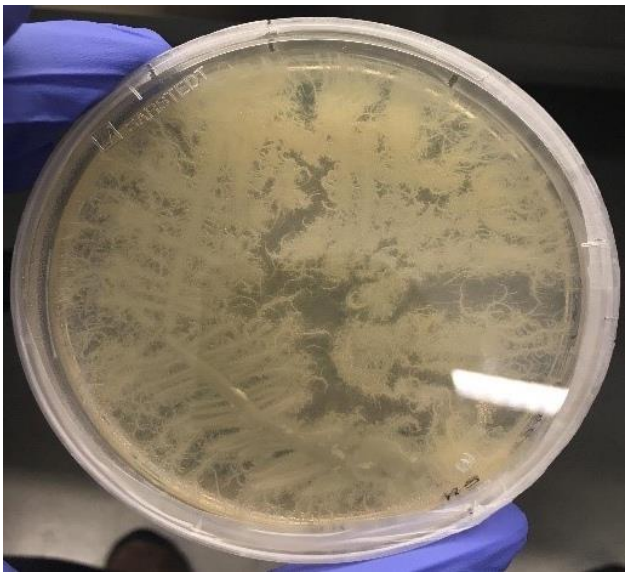

Figure 3. Actinomycetes from soil print.

Next step was to do tissue prints of plant roots, with the expectation to find bacteria clearly associated with plants. Roots from 7 different wild plants grown in undisturbed environments were harvested. They were chosen based on phenotypic similarities with *Solanum tuberosum,* but also a closer phylogenetic relationship. Before the roots were placed on LB-plates, excess soil was washed off with water.

Many of the root-associated bacteria that appeared seemed to be varieties of actinomycetes (Figure 3), hence nothing that we regarded as novel. We were applying two simple visual criteria for a successful candidate, a bacterium that showed aggressive colonization all over the root surface and the production of biofilm. Further, it would also be an advantage if the host plant belonged to the *Solanum spp*.

Finally, a tissue print that met all these three criteria was found (Figure 4). It visually seemed as a dominant root associated bacterium that covered all of the LB agar plate with a fairly large amount of surrounding biofilm. The roots belonged to a plant commonly referred to as bittersweet (*Solanum dulcamara*). An example of phylogenetic relationships based on sequencing of tropinone reductase genes is shown in Figure 5.

Figure 4. Tissue print of roots belonging to *S. dulcamara*. showing dominant and homogenous colonies all over the root surface with a distinct biofilm surrounding the colonies.

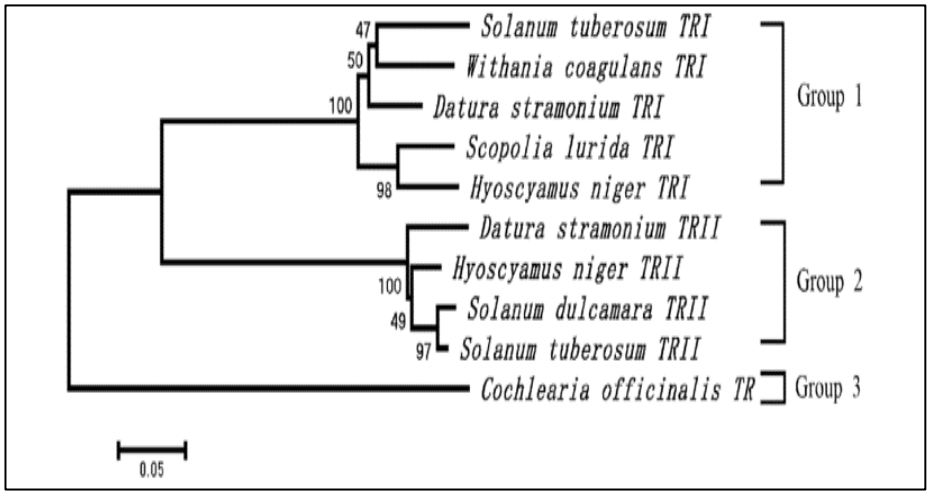

Figure 5. Phylogenetic analysis based on plant tropinone reductases. (Zhao et al, 2017)

The bacterium was then cultivated in liquid LB at 28 °C. After 3 days the solution was estimated to be somewhat saturated. Before greenhouse trials were initiated, a test was made to study if the bacterium could colonize potato plants. Tubers were inoculated with bacteria, planted and then parts from the upcoming plants were collected for tissue prints on LB agar.

Pre-sprouted potato tubers (variety: Sevilla) were inoculated with bacteria and then planted in small growth chambers in the phytotron using four different concentrations (10^6, 10^7, 10^8, 2.5 x 10^8 CFU/ml) of bacteria with, what at this point, was considered one single strain of bacteria. The bacteria were in LB solution and accordingly the control group was treated with LB solution alone. Then the plants grew for approximately one week before the collection of different plant tissues, i.e. roots, shoots, leaves and tubers were made. The tissue prints were incubated at 28°C.

After about 24 h visual examinations of the tissue prints of bacteria inoculated tissues and the control group were made. A strong indication of visual differences was apparent and confirmed by the co-supervisor and microbiologist Mikael Pell (Figure 6 and 7).

Although this screening was not a perfectly controlled or well-documented part of the project, it is described as it gave some indication of the bacteria's ability to colonize potato plants.

Figure 6. Visual differences observed during screening tissue prints from upcoming tubers inoculated with bacteria, as well as a control group with medium only.

Figure 7. Tissue prints of potato root. Bacterial inoculation of tuber to the left. Control sample to the right

However, the bacteria did not display the same appearance on these tissue prints as the earlier tissue prints made of Bittersweet roots. Although it looked like the bacteria from the CFU series, which simply lacked the distinct appearance of single colonies with surrounding biofilm.

As the work continued with preparation of various stock solutions and concentration measurements by CFU counting, it became obvious that the colonies varied slightly in size and colour. This indicated that there might be two different strains/types of bacteria. Therefore, the two types of colonies were separated, and two new cultures started to make stock solutions (Figure 8). The two strains are referred to as Large (L) and Small (S) reflecting the colony sizes.

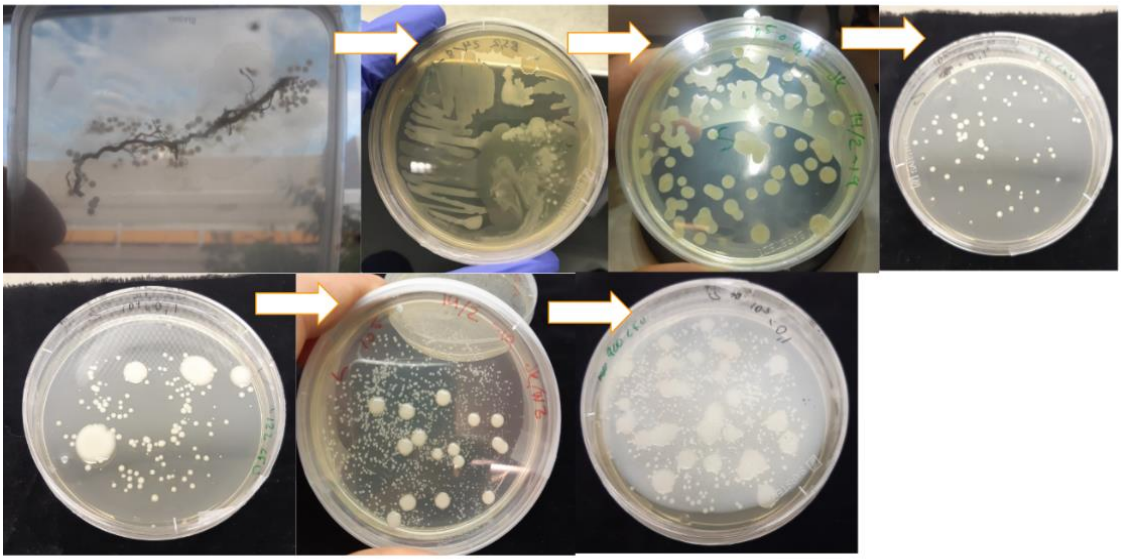

Figure 8. Pictures illustrating the changing structure and appearance of the bacteria at different time points of cultivation on LB-agar

In a previous experiment the bacterial stock solution was heat shocked at 60 °C during 10 minutes to investigate whether the bacteria had the ability to produce spores or not. The main reason for doing this was to study the ability to sporulate since spores are advantageous for seed treatment. For instance, the Swedish company BioAgri use the bacteria *P. chlororaphis* for seed coating but this bacterium lacks the ability to produce spores and therefore has to be applied in a special formulation in order to keep the bacteria alive during storage, but the shelf life is still limited. If spores were available, this would simplify the application of the bacterial coating, as well as guarantee long time storage of treated seeds. On the other hand, the ability to produce spores also implicates the ability to persist in the soil, which can be regarded as both positive and negative. On one hand the beneficial bacteria can become a part of the soil microflora (if not already present), on the other hand it might affect the ecology of the soil. It is difficult to judge if this is good or bad, although important to investigate.

Spore producing bacteria can withstand temperatures to at least 90 °C (Pell and Passoth, 2018). The heating bath in the microbiology lab only reached 70-80 °C, which instead was run for 20 minutes (Figure 9). After centrifugation and washing of the bacterial pellet in phosphate buffered saline (PBS), it turned out to be very difficult to obtain a homogenous suspension. A big lump of what was considered as denatured bacterial material was obtained, so it was concluded that the bacteria did not sporulate (Figure 10).

Figure 9. Heat shock treatment of bacteria in heating bath at 70 °C for 20 minutes.

Figure 10. Denaturation/precipitation after heat shock-treatment at 70°C for 20 minutes), indicating non-spore producing ability.

In order to generate a functional stock solution a new culture, without subsequent heat shock, was started in LB at 28 °C for about 62 hours. The bacteria were centrifuged (5000 rpm) followed by washing of the pellets in PBS. The concentration was decided by CFU counting. The workflow is depicted in Figure 11.

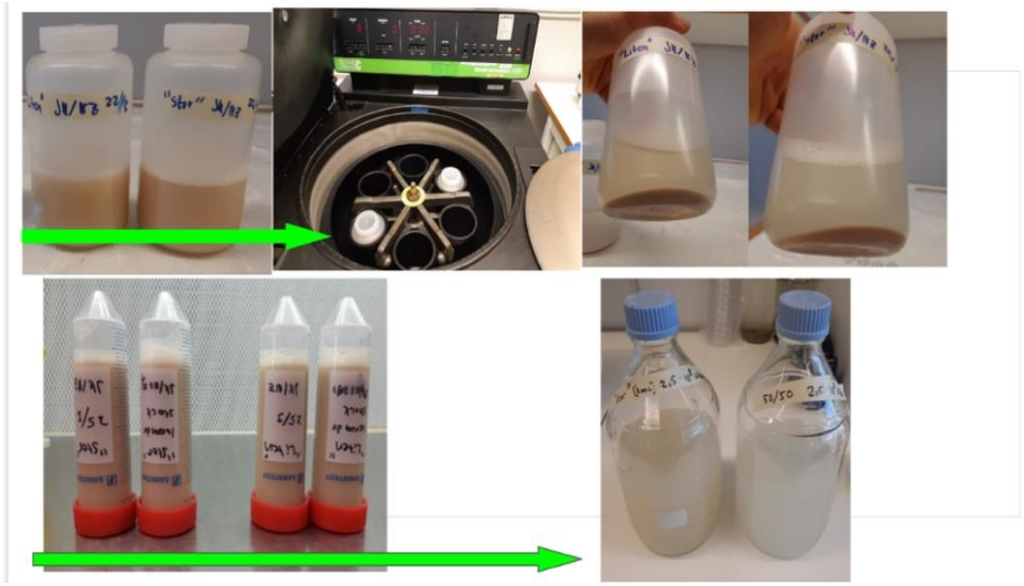

Figure 11. Workflow of bacteria purification. From bacterial culture in LB, to centrifugation to bacterial pellet washed in PBS and final suspension in tap water (the experimental working solution).

3 Plant growth experiment

Potato variety: Asterix (certified tubers)

Since potato tubers vary a lot in different parameters the tuber material (cv. Asterix) was scored before the growth experiment was initiated. Phenotyping of the tubers included factors of measurable quality, such as the weight of the tubers, the number of sprouts on each tuber, the number of eyes per tuber, and the number of upcoming shoots after planting the material.

These three variables were measured and scored pre-planting, and the tubers were divided into 3 groups submitted to 3 different treatments (control, “50/50” mix of bacteria and bacterium “Large”), with 16 tubers in each group with the aim to minimize the variability in mass, which was considered as the most critical and easily measurable variable (Figure 12). Later, the three groups were randomly selected for which treatment they should receive.

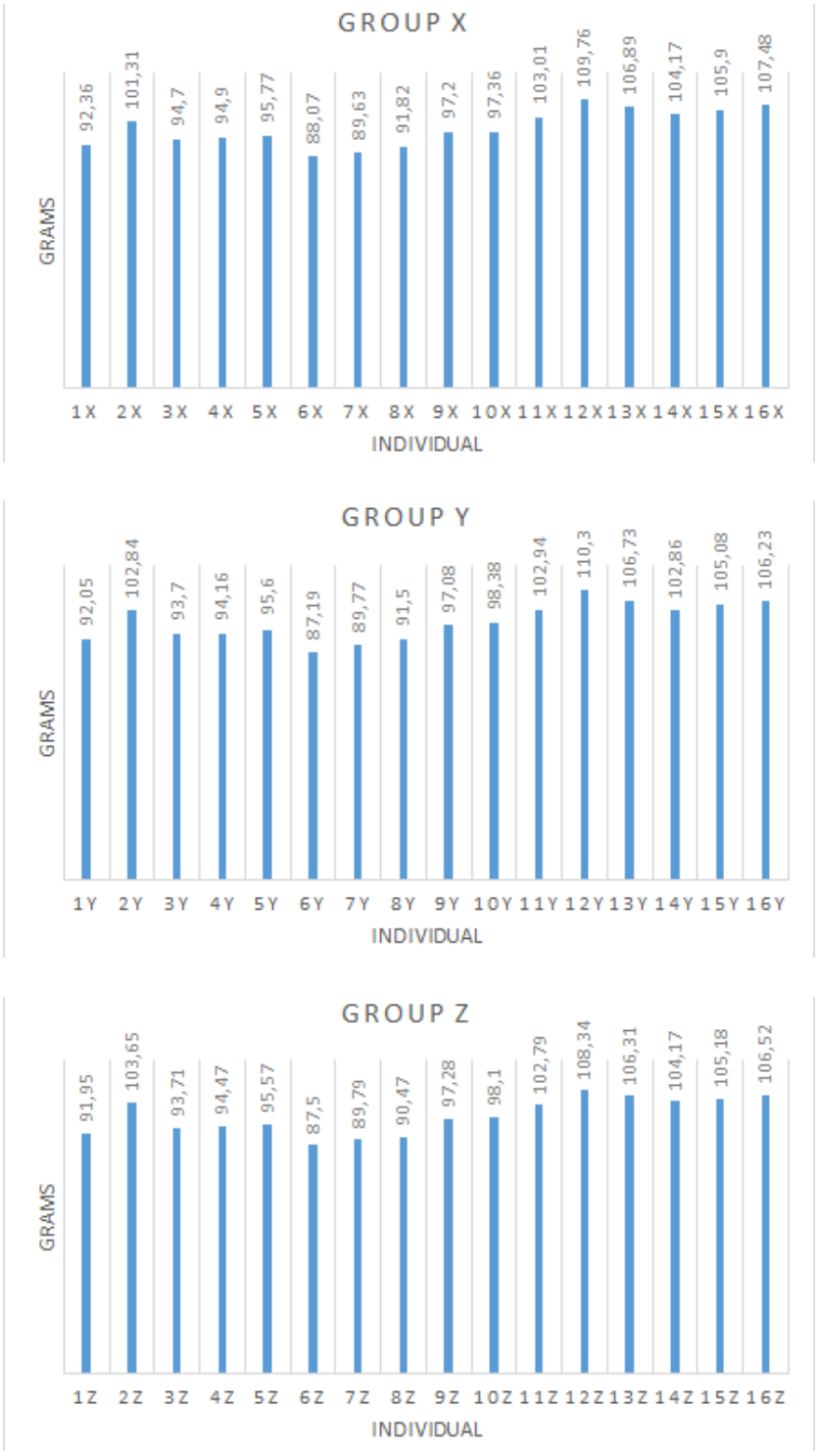

Figure 12. Individual tuber mass in the control group (Y), the group inoculated with "50/50" bacteria mix (X) and the group inoculated with bacterium "Large" (Z). This sorting of the tubers was made to minimize variation. Each group was the randomly assigned to get one of the three possible treatments.

How could these measurements be applied for statistical analyses in relation to post-harvest analysis? Shoot were considered geometrically similar to cylinders, and the volume of shoots from each plant was calculated. It was considered to be a simple way to create linear measurements to correlate with the above soil biomass of each plant, just after emergence. This was to be a “shoot-biomass-index”.

There was three treatments with 16 tubers each. One control group “Y” (treated with water) and two groups with bacterial inoculation “X” (“Large” 1.25 x 10_8 CFU/ml + “Small” bacteria 1.25 x 10_8 CFU/ml) and “Z” (“Large” bacteria, 2.5 x 10_8 CFU/ml).

The inoculation was made by soaking tubers in solutions with shaking for 2 hours to avoid settling of bacteria (Figure 13).

Figure 13. Treatment of the tubers with the 50/50-mix of bacteria (left), water (center) and bacterium “Large” (right) on a shaker to prevent the bacterial cells from settling while soaking the tubers in the solution.

The tubers were planted in pots with prefilled soil at a depth of 7 cm. Each pot got exactly the same weight of soil with the same moisture. After 9 days all shoots had appeared, and the number of shoots and the length and width of each shoot were measured on each individual to understand the potential of each tuber (Figure 14). This analysis was performed to obtain a flat level that was based on genetic variation rather than the effect of the treatment. The assumption was that a potential effect due to treatment would increase over time.

Figure 14. Plant growth-promotion experiment in the greenhouse showing shoot development of the tubers with different treatments after 7 days (left) and 9 days (right).

3.1 Results

The tubers were harvested after 6 weeks in the greenhouse and divided into three categories (seed tuber, new tubers and shoots). The reason for taking the seed tubers into account was the short growth period, which in many cases meant that the tubers were not consumed. Figure 15 displays the biomass of three harvest components that were measured (seed tuber, above soil biomass and new tubers). Only the above soil biomass had the dry weight measured for each individual in respective group. The reason for three harvest components being measured was due to premature harvest (after 6 weeks), hence the biomass was not fully accumulated to the new tubers. Overall there was no significant differences between the treatments with regards to dry weight.

Figure 16 illustrates the number of new tubers for every individual for each group, respectively, as well as which individuals that had the seed tubers left. Although no statistically adequate analysis could be made (because of no individual dry weight of the newly formed tubers was performed), an interesting observation was made. It was about the ratio between the dry and wet weight of the new tubers as pooled individuals from each group that had their seed tubers still intact. The following ratios were observed: group inoculated with "50/50" mix of bacteria (X) = 0.269 (10 individuals), group inoculated with bacterium “Large” only (Z) = 0.343 (8 individuals) and the control group (Y) = 0.238 (11 individuals). If these individuals were pooled together (based on the criteria that they had their seed tuber intact), one could observe a substantial difference between group Z compared to X and Y. The ratio between dry- and wet weight is 44 % larger in group Z compared to group Y, and 27.5 % larger in Z compared to X.

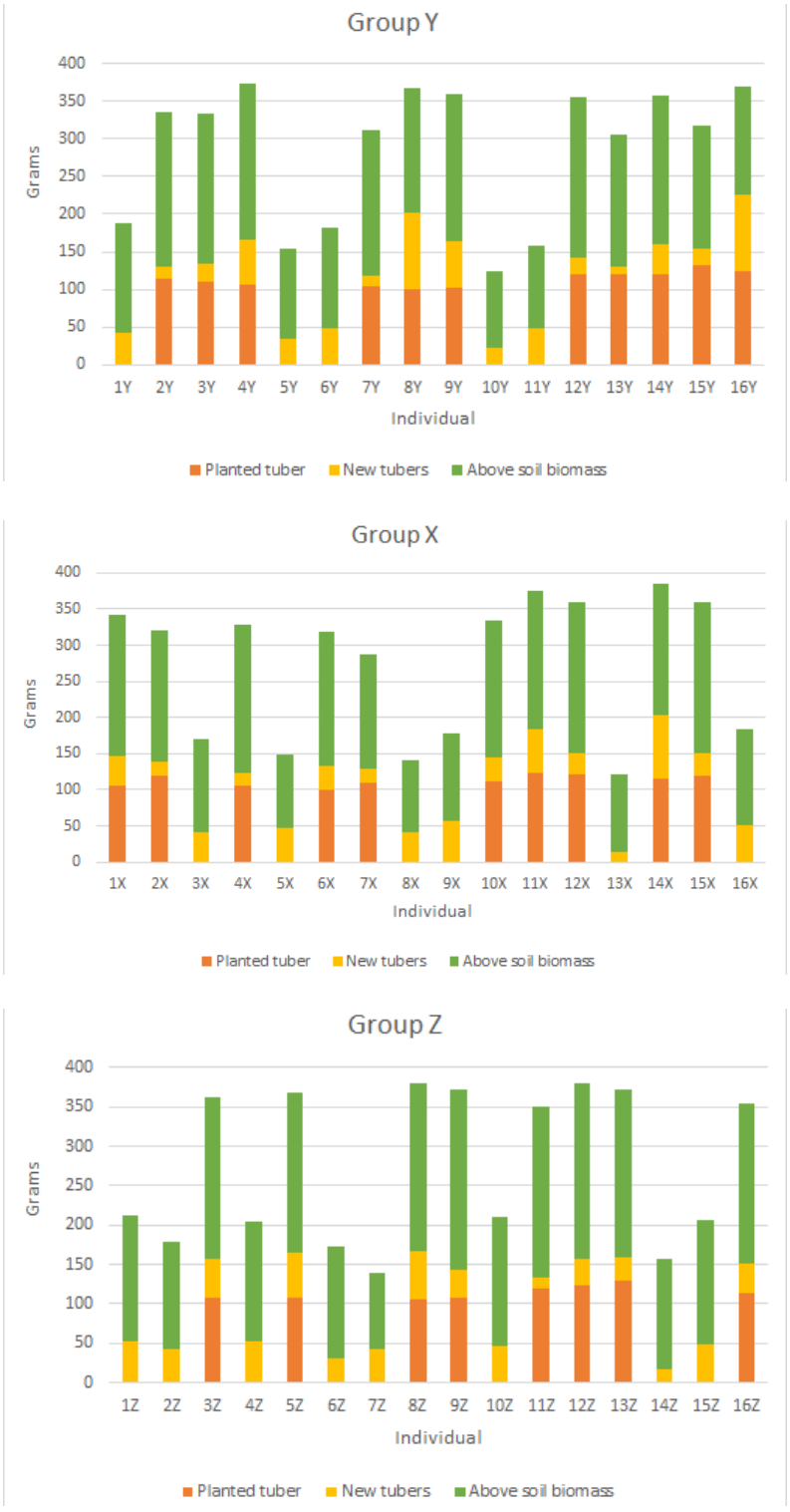

Figure 15. Harvest components from growth-promotion experiment in the control group (Y), tubers inoculated with a "50/50" mix of bacteria (X) or (Z) with tubers inoculated with "Large" bacteria only.

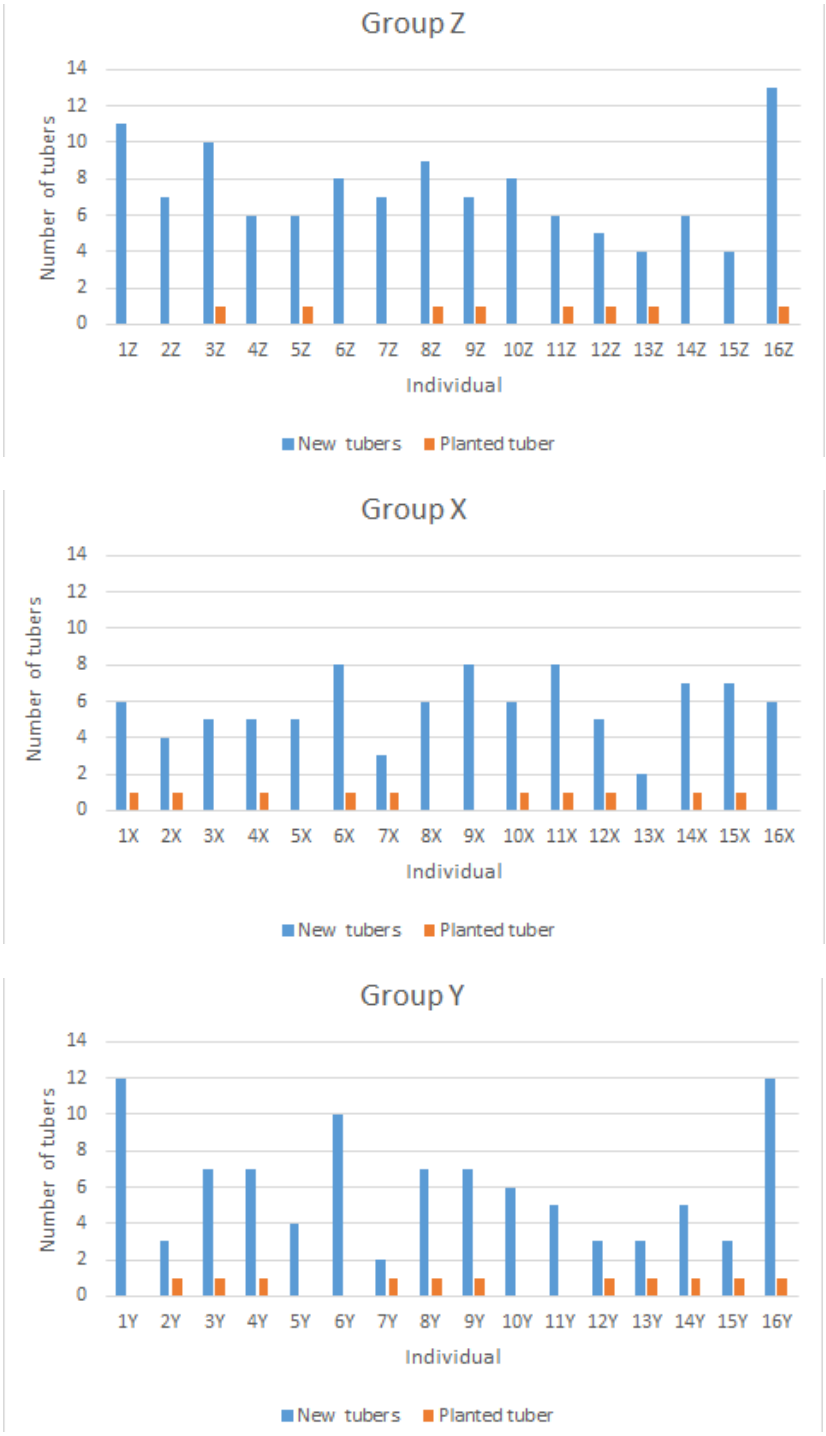

Figure 16. Number of seed tubers (Planted tuber), and newly formed tubers from growth-promotion experiment in the control group (Y), the group inoculated with the "50/50" mix of bacteria (X) and the group inoculated with bacterium "Large" only (Z).

With regards to the above soil wet biomass, statistically significant differences were observed between group Z (bacterial treatment, ”Large”) and group X (bacterial treatment, ”Small”/”Large”) and group Y (Control) (Figure 17, Table 2) (only the individuals with the planting tubers left were taken into account). Group Z above soil wet biomass was about 14% larger compared to X and Y. When all individuals were included no significance was at hand.

There was also a significant difference in the actual water content of above ground biomass between group Z and X (Figure 18, Table 3). Although the difference was only about 1 %.

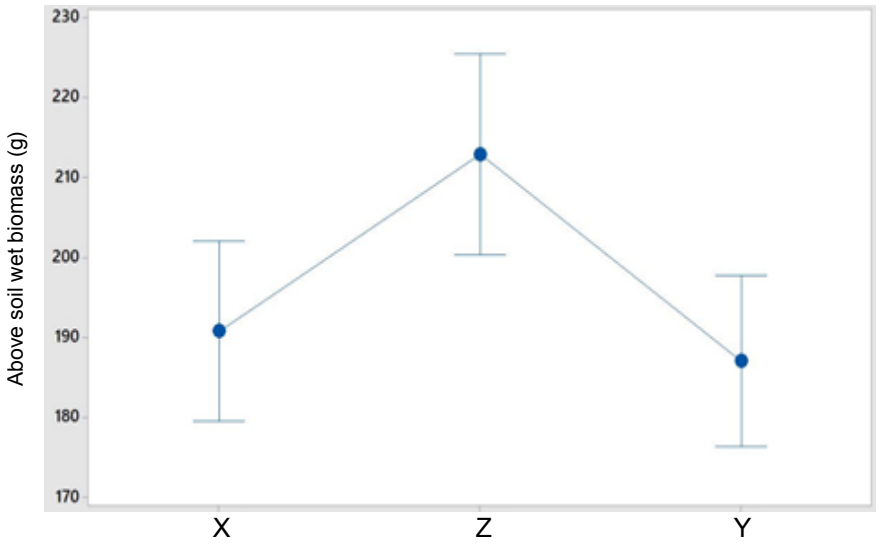

Figure 17. Above soil wet biomass from growth-promotion experiment in the group inoculated with the "50/50" mix of bacteria (X), the group inoculated with bacterium "Large" only (Z) and the control group (Y). Only samples with seed tubers left included.

Table 2. ANOVA of aboveground wet biomass. Only samples with seed tubers left included (to make a comparison with less variation with regards to the other harvest components).

Factor	N	Mean	Grouping*
X	10	190.78	B
Y	11	187.03	B
Z	8	212.88	A

**Means that do not share the same letter are significantly different*

No significant differences of growth were found between the two bacterial treatments (Z, X) and the control group (Y) with regard to dry weight of the harvested new tubers (data not shown).

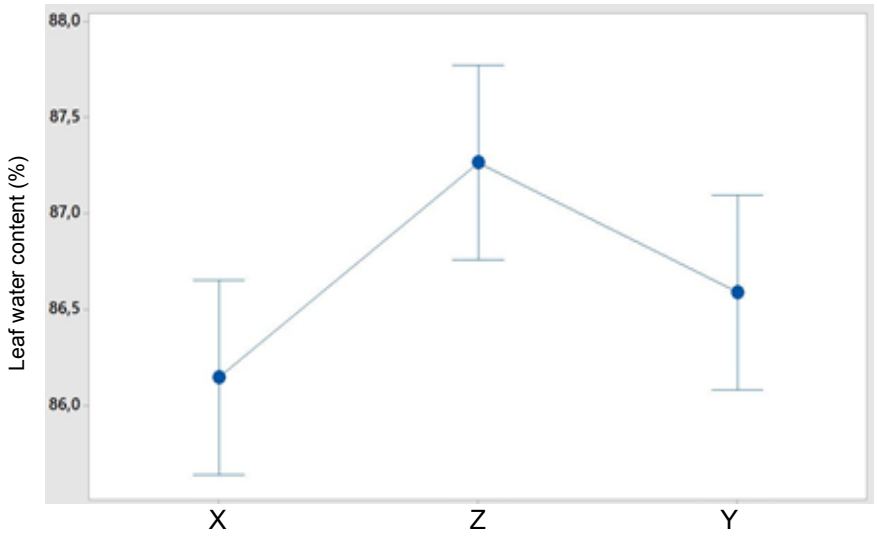

Figure 18. Leaf water content from growth-promotion experiment in the group inoculated with the "50/50" mix of bacteria (X), the group inoculated with bacterium "Large" only (Z) and the control group (Y). All samples included.

Table 3. ANOVA of water content of leaves. All samples included.

Factor	N	Mean	Grouping*	
X	16	86.144		B
Y	16	86.587	A	B
Z	16	87.262	A	

**Means with different letters are significantly different*

Fresh weight of the new tubers did not differ significantly between the groups either if all individuals were included or only those that with the planting tubers left were taken into account (Figure 19*, Table 4*).

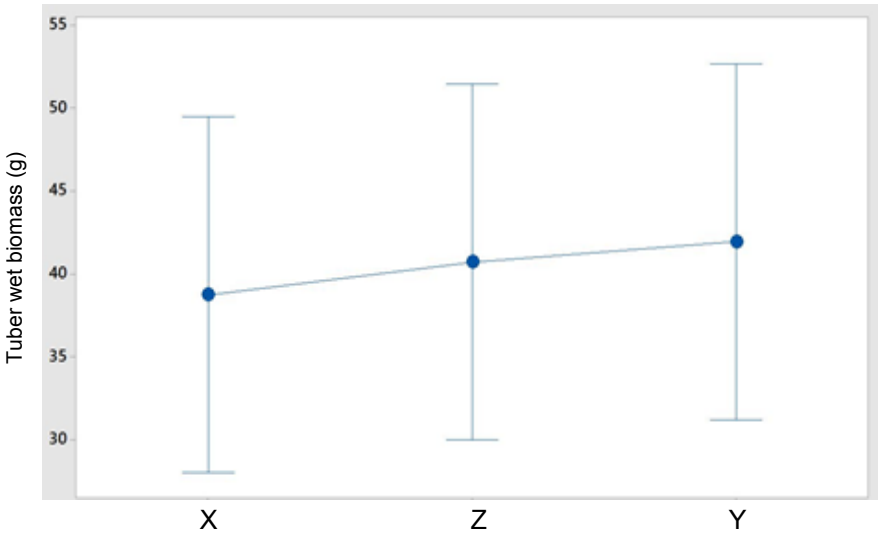

Figure 19. Tuber wet biomass from growth-promotion experiment in the group inoculated with the "50/50" mix of bacteria (X), the group inoculated with bacterium "Large" only (Z) and the control group (Y). All samples included.

Table 4. ANOVA tuber wet mass new tubers. All tubers included.

Factor	N	Mean	Grouping*
X	16	38,73	A
Y	16	41,93	A
Z	16	40,70	A

**Means with different letters are significantly different*

3.2 Discussion

No major significant differences with regards to dry weight of the biomass components were observed between the three different treatments. One interpretation is that the bacteria simply did not have any growth promoting qualities or did not manage to colonize the plant material. It could also be a combination of these two factors. Other possibilities are that an optimal dose or application mode was not used.

Different cultivars were used in the initial screening (Sevilla) and the final experiments (Asterix). The cultivars also differed in terms of presprouting, peel thickness and structure, but also in time to harvest. Asterix tubers were not pre-sprouted to be more representative of real farming practices. Since the material was not pre-sprouted it took about 8 days from the time of planting to the time of appearance for the first upcoming shoots. This increases the likelihood of the bacteria being outcompeted by the soil microbiota if not having high rhizosphere competence.

In the screening process,we inoculated the tubers with bacteria directly in LB solution, while the control was treated with LB without bacteria. The use of LB during inoculation is also a possible source of error since LB is a complex nutrient rich medium, which possibly can affect the plant material. When applying the bacteria in LB solution, about the same concentration (2.5 x 10_8 CFU/ml) was used as when applying the bacteria in water solution. Since the viscosity of LB is higher than that of water, one could expect a difference in adhesive ability affecting the colonization efficiency. Another factor is bacterial survival that probably favors LB over water.

It is still possible that the bacteria colonized the tubers followed by the roots but did not have any noticeable effect. If that was the case, the bacteria may be regarded as commensals.

If time had allowed, we would have done new tissue prints right after harvest to observe if the bacteria had colonized, survived and multiplied. Since it is possible that the bacteria might become endophytic, one should have done the tissue print differently or tested other methods. An endophyte applied to a vegetative part of a plant, such as a tuber, might not be the same as the application to a seed. One could assume a more successful colonization process if the tubers would have been pre-sprouted. A more sensitive method (without spatial resolution) to confirm colonization would be to perform real time PCR of roots based on specific bacterial gene markers. Since neither time nor genetic markers were at hand, such experiments could not be performed.

Another possible scenario is that the bacteria might show general PGPR qualities if they were to be screened *in vitro*. However, under natural conditions, positive effects may perhaps only appear under very specific conditions (plant material, soil and climatic conditions etc).

As discussed, one possible scenario is that the two bacteria could have growth promoting qualities with the indirect mechanism of suppressing soil borne disease under field conditions. However, since we used commercial soil that should be free from larger amounts of plant pathogens, this putative effect would not be possible to observe during our growth experiment.

The time of harvest was very early which might have affected the outcome of the results.

4 *P. infestans* Infection of Potato Tubers inoculated with the Bacterial Strains

4.1 Materials & Methods

The potato variety King Edward was used because of its susceptibility to *P. infestans*. The tubers were treated by spraying water or the 50/50 mix of bacteria and bacterium 'Large' in water solution. The treatment specifications were the same as in the growth-promotion experiment (Table 5).

Table 5. *Overview of treatments for different groups for the biocontrol experiment with P. infestans bacteria*

	Treatment
Control group	Sprayed with water
50/50 bacteria mix	Sprayed with a 1:1 mixture of 'Large' and 'Small' (1.25 10_8 CFU/ml each)
Bacterium 'Large'	Sprayed with a solution of 'Large' (2.5 x 10_8 CFU/ml)

Spraying was performed in a fume hood to avoid cross-contamination and inhalation of aerosols. The tubers were left to dry for approximately 4 hours.

The *P. infestans* strain 41 used is considered to be a new, very virulent variety. The strain was cultivated on rye-pea agar and inoculated on potato slices to obtain sporangia. Five days later, sporangia were harvested by washing off into a suspension. The sporangia concentration was calculated to 10_4 sporangia/ml using a haemocytometer. This suspension was kept at 4°C for 2 hours to enable the sporangia to hatch into zoospores.

The control group and the treated groups were placed in a separate Ziplock bag with about 25 tubers each before adding 25 ml of 10_4 spores/ml (Figure 20). After 4 days the excess liquid was removed and the tubers were left in the bags for an additional 6 days.

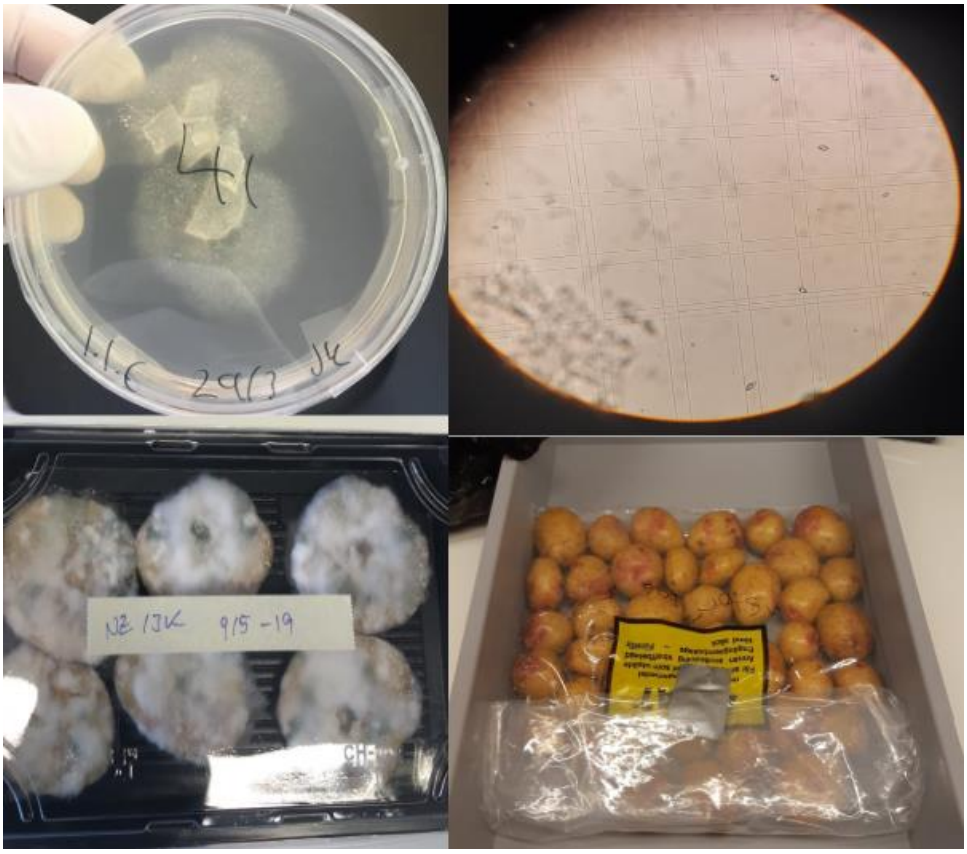

Figure 20. Pictures from tuber experiment with *P. infestans* cultivates (upper left), *P. infestans* spores (upper right), potato slices infected with *P. infestans* (lower left) and the Ziplock bag with potato tubers to be infected (lower right).

4.2 Results & Discussion

At the end of the experiment (approximately 10 days) the tubers were almost rotten but no disease symptoms due to *P. infestans* were visible in any of the samples from any of the three groups. The experiment was not successful with respect to disease development and potential protection by bacteria could thus not be studied.

One possible explanation is that the virulence of the *Phytophtora* isolate used was not high enough. Time constraints did not allow virulence tests beforehand. Another possibility is that the environment was suboptimal for the development of the potato late blight. As earlier attempts to cultivate ‘Small’ and ‘Large’ on potato slices without the presence of moisture were unsuccessful we decided to only remove excess fluid from the tuber bags instead of drying them completely. This might have inhibited the growth and development of *P. infestans* on and inside the tubers as the zoospores do not become infectious under such conditions. The environment instead seemed to benefit the growth of ‘Small’ and ‘Large’ which

probably further worsened conditions for *P. infestans*. A check by microscopy to verify that the sporangia in the solution obtained from the potato slices had burst should have been conducted as well to be able to exclude the absence of zoospores as a source of error

An advanced state of decay was observed in 35% of the tubers treated with a 50/50 mixture of 'Small' and 'Large' whereas only 22.5% of the tubers treated with 'Large' were in an advanced state of decay. In the control group treated with water, 24% of the tubers were in an advanced state of decay (Table 6). Additional experiments would be necessary to establish whether these differences are significant and reproducible and whether 'Small' or the combination of 'Small' and 'Large' causes the increased number of affected tubers.

Table 6. *Number of decayed tubers per group for the biocontrol experiment with P. infestans and bacteria*

	# of tubers	# of decayed tubers	Fraction of decayed tubers
Control group	37	9	24.3%
50/50 bacteria mix	40	14	35.0%
Bacterium 'Large'	40	9	22.5%

5 *P. infestans* and *A. solani* Infection of Potato Leaves inoculated with the Bacterial Strains

5.1 Materials & Methods

The purpose of the leaf experiment was to determine possible antagonistic interactions between our bacteria and two common pathogens of potatoes, *P. infestans* and *A. solani*. For this experiment *P. infestans* was cultivated on potato slices of the variety King Edward. *A. solani* was cultivated on potato-dextrose agar (PDA). Healthy looking leaves were cut off from plants from the PGPR experiment and treated with water, the 50/50 mix of bacteria or bacterium 'Large' in accordance to the plant group they were taken from (Table 7).

Table 7. *Overview of treatments for different groups for the biocontrol experiment with P. infestans, A. solani and bacteria*

	Treatment
Control group	Submerged in water
50/50 bacteria mix	Submerged in a 1:1 mixture of 'Large' and 'Small' (1.25 10_8 CFU/ml each)
Bacterium 'Large'	Submerged in a solution of 'Large' (2.5 x 10_8 CFU/ml)

After treatment the leaves were put onto plates with water agar and incubated for 3 days in a 16/8 light-schedule (22 °C in light, 20 °C in dark). At the end of the incubation period each group Mix, Control, 'Large' was randomly divided into 3 subgroups of which one was not inoculated with any pathogen (-C), one was inoculated with *P. infestans* (-P) and one was inoculated with *A. solani* (-A) (Table 8).

Table 8. *Overview of treatment combinations for the biocontrol experiment with P. infestans, A. solani and bacteria*

	Control	*P. infestans*	*A. solani*
Control group	Control-C	Control-P	Control-A
50/50 bacteria mix	Mix-C	Mix-P	Mix-A
Bacterium 'Large'	Large-C	Large-P	Large-A

The inoculation method for *P. infestans* was based on (Grönberg et al. 2011). One 20 µL droplet of sporangial solution containing 15 x 10_4 spores was placed on each side of the middle vein on the abaxial side of the leaf. The leaves were subsequently incubated upside down.

For inoculation with *A. solani*, discs with a diameter of 7 mm were punched out of PDA plates previously inoculated with *A. solani* strain 5245. The discs were placed on the leaves at the same position as the sporangial solution with *P. infestans* and pressed onto the leaves (Figure 21).

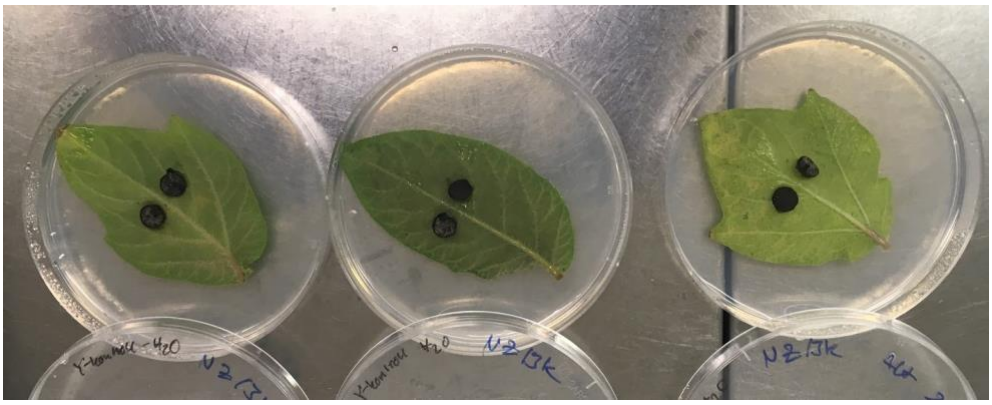

Figure 21. Position of the *A. solani* pathogen on leaves

Since inoculation for the pathogen control groups (Mix-C, Control-C and 'Large'-C) would have been biased towards *P. infestans* (treatment with water) or *A. solani* (treatment with a sterile agar disc), the leaves were just turned upside-down without any further treatment. All groups of leaves were incubated for 10 days in a 16/8 light-schedule (22 °C in light, 20 °C in dark).

5.2 Results & Discussion

Due to time limitation, the fast deterioration and the poor condition of the leaves at the end of the experiment, a detailed grading scheme for the level of infection was deemed inappropriate. It also proofed to be difficult to verify that the observed deterioration of specific leaves had been caused by our bacteria, one of the

pathogens, unintended infections with other bacteria or pathogens or physical damage. Instead, the healthiness of the leaves between different groups was judged visually by several persons with different levels of knowledge about the groups and the experiment itself. The material was coded before screening so the true identity of the groups was not known. However, the method of pathogen application for the leaves inoculated with *A. solani* made them easily distinguishable from the group inoculated with *P. infestans* and the control group.

The findings should be regarded as an indication to which approach may yield interesting results in future experiments rather than proof or disproof of bacteria – pathogen interaction. We recommend that the plants the leaves are taken from are grown in a protected environment which was not the case here as they were grown in the greenhouse with other plants and possible pathogens. When cutting of the leaves, sterile tools should be used and part of the stem of the leaves should be removed under sterile conditions as we suspect that the damage in some of the leaves spread from the leaf-stem. The method of inoculation with *A. solani* should be reviewed as it did not seem to work for all samples.

5.2.1 *P. infestans* vs. control

The control group without bacteria and without pathogen (Control-C) appeared as the healthiest one. Interestingly the group with both the 50/50-mix (Mix-P) and *P. infestans* was graded second healthiest, while the group containing both 'Large' and *P. infestans* ('Large'-P) was rated least healthy. The group containing only one of either bacterium/bacteria mix or *P. infestans* (Mix-C, 'Large'-C and Control-P) was rated in between in different order by different people. Apparently, the bacteria were as pathogenic for the leaves as the pathogen itself in the concentration tested. This was unexpected as an earlier test had shown that the bacteria had no negative effect on the leaves.

'Large' and *P. infestans* seemed to be able to co-exist and cause more damage to the leaves together than each on its own. This could be an indication that they exploit different niches or stimulate each other to do more harm.

The 50/50-mix and *P. infestans* exhibited a different behaviour, the combination caused less damage than each on its own. This, in turn, could mean that they are competing for the same niche or are inhibited by the presence of the other one.

5.2.2 *A. solani* vs. control

Two samples (one from group Control-A and one from group Mix-A) did not show any signs of infection with or growth of *A. solani* and were therefore disregarded since we believe that inoculation was not performed correctly.

Among the remaining samples, no notable difference was observed between the different groups infected with *A. solani* (Mix-A, Control-A and 'Large'-A) and almost all samples infected with *A. solani* looked less healthy than the corresponding control samples (Mix-C, Control-C, 'Large'-C).

6 Identification and classification of bacteria

6.1 Introduction

6.1.1 16S rDNA sequencing

Thanks to technical advances and increasing knowledge of the structure and function of genes, the generation of reference material in DNA databases, gene sequencing has become an integral part of identifying the nature of unknown specimens. Protocols for gene sequencing differ slightly between different areas of application. The common procedures include isolation of cells, DNA extraction or purification, DNA fragment amplification and sequencing followed by data analysis and comparison to reference sequences.

To definitively identify a species through DNA analysis, whole genome sequencing is ideally required (which was out of scope in this project). Apart from the workload and cost involved in the preparation and sequencing process, the identification process demands a lot of computing power and relies on the availability of high-quality reference sequences as well as some complementary work to characterize potential gaps and repetitive regions.

A less expensive and less time-consuming technique to classify and identify organisms is targeted amplicon sequencing. The most common strategy to study bacteria is the use of 16S ribosomal RNA (rRNA) gene sequencing. Woese and Fox (Woese and Fox 1977) ascertained as early as 1977 that rRNA is a suitable marker to distinguish different species and establish their relationship to each other from an evolutionary point of view, i.e. phylogeny, without the need to sequence whole genomes of bacteria. The construction of these phylogenetic relationships is based on the fact that rRNA is a fundamental component of all living organisms and that the corresponding genes are relatively conserved between different species due to

the low rate of mutation in certain parts of this gene (Janda and Abbot 2007). The rRNA sequences in regions involved in attachment of tRNA species, mRNA or subunit interaction are for example more conserved while gene sequences in intervening regions are more variable. The 16S rRNA gene contains sites where universal oligonucleotide primers used for polymerase chain reaction (PCR) analysis can bind. Intersecting these more conserved primer-specific binding-sites are hypervariable regions that differ between different bacteria and can be used for identification to a certain taxonomic level (Figure 22).

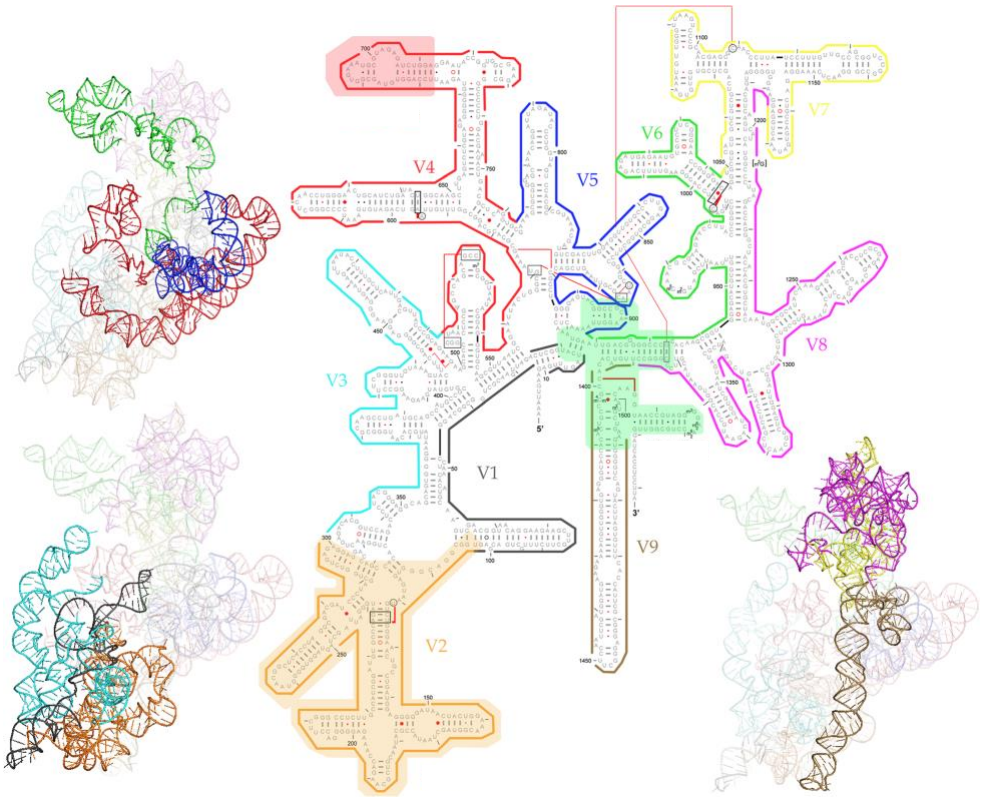

Figure 22. The 16S rRNA gene with variable regions V1 to V9 coloured in 2D and the 3D-structure (adapted from Yang et al. 2016).

Using a suitable combination of forward and reverse primers, different parts of a bacterial 16S rRNA can be amplified and sequenced. Bacteria most often possess more than one copy of the 16S rRNA gene. Due to mutation, these copies are not necessarily identical which can make it difficult to align sequences of known genes to gene sequences of the unknown sample.

Another approach for the identification of bacteria is sequencing of other specific genes where so-called housekeeping genes are commonly studied (Martens et al.

2008). Such genes are required for basic cellular functionality e.g metabolism of amino acids and sugars, DNA replication and RNA synthesis. This approach can be used on its own or in combination with 16S rRNA gene sequencing.

6.1.2 Biotests

Bacteria within the same genus or even the same family can have almost identical hypervariable regions within the 16S rRNA gene and very similar sets of housekeeping genes. If complete genome sequencing is not feasible, additional methods for identification are used to narrow down the number of possible alternatives. Biological and biochemical tests, or biotests, are inexpensive and fast methods to complement above mentioned gene sequencing techniques. These biotests can for example establish whether a bacterium is gram-positive or gram-negative, which substrates it can use as fuel for growth and which enzymes or by-products that are created in this process. Biotests can also differentiate bacteria based on the ability to reproduce in aerobic and anaerobic environments, adapt to different temperatures or withstand saline and other stressful conditions. Analytical profile indexing (API) is one of the commercially available solutions for identifying specific groups of bacteria based on their ability to process different types of substrates. A predefined concentration of bacterial cells suspended in a medium is applied to different reagent wells containing different substrates and an indicator dye. If a bacterium is able to digest a certain substrate the colour of that well changes (Figure 23). The specific pattern of the ability to digest certain substrates can then be compared to reference matrices provided by the company producing the API test or earlier research (Table 9 and Figure 24).

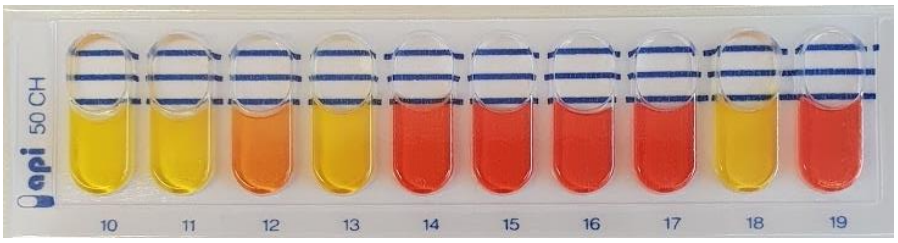

Figure 23. Example of an API test strip where the initial red/orange of the medium (well 16) changes to different shades of orange/yellow. A more intense yellow indicates a higher ability to digest a certain substrate.

Table 9. *Excerpt of the API test result for bacterium 'Large' for sugar utilization*

Substrate #	Substrate Name	
10	D-Galactose	++++
11	D-Glucose	++++
12	D-Fructose	++
13	D-Mannose	++++
14	L-Sorbose	-
15	L-Rhamnose	-
16	Dulcitol	-
17	Inositol	-
18	D-Mannitol	++++
19	D-Sorbitol	-

Sometimes an online identification solution is available, where the sample matrix can be entered directly and comparison and best-hit analysis is performed automatically providing a tentative or probable identification.

	Stenotrophomonas -stam							
Substrat	**CCG 57/1 röd**	**CCG 57/2 orange**	**CCG 57 Slimy 1 röd**	**CCG 57 Slimy 2 orange**	**CCNC 87/1 röd**	**CCNC 87/2 orange**	***S. mal***	***S. rhi***
D-Galaktos	-	-	-	-	-	-	-	-
D-Glukos	+	+	+	+	+	+	++	++
D-Fruktos	+	+	+	+	+	++	++	+
D-Mannos	+	+	+	+	++	++	++	++
L-Sorbos	-	-	-	-	-	-	-	-
L-Rhamnos	-	-	-	-	-	-	-	-
Dulsitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-
D-Sorbitol	-	-	-	-	-	-	-	-

+ = svag tillväxt, ++ = tydligt tillväxt, +++ = mycket rik tillväxt och s = mycket svag och svårt urskiljbar tillväxt.
S. mal = *S. maltophilia* DSM 50170, data från detta arbete.
S. rhi = *S. rhizophila* DSM 11405, data från detta arbete.

Figure 24. Example of API test results of different *Stenotrophomonas* strains from literature (adapted from Klingenberg 2011)

Even though many companies only supply identification matrices for a few known species or families, an API test can provide valuable information about a bacterium as the test usually contains several different substrates and knowledge about the bacterium-substrate compatibility can be used for manually identifying bacteria or narrowing down the number of possible alternatives based on biological characteristics.

6.2 Materials & Methods

6.2.1 Gene sequencing

DNA Purification

For DNA purification, the readily available Thermo Scientific GeneJET Genomic DNA Purification Kit was used. The following protocol is based on the instructions included in the kit (Thermo Scientific 2016a) Since gram-staining was not performed before the DNA purification, the protocol for gram-positive bacteria was used as it is able to handle the thicker mucus layer on gram-positive bacteria without having a negative effect on gram-negative-bacteria.

In accordance to the protocol instructions a lysis buffer for gram-positive bacteria containing 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100 and 20 mg lysozyme/mL buffer solution was prepared. This buffer is necessary to dissolve cell walls of gram-positive bacteria.

Two replicates of 1.5 mL sample containing up to 2 x 10_9 bacterial cells were harvested of each bacterium, 'Large' and 'Small' and at two different concentrations (T and Y) resulting in a total of 8 samples. The Eppendorf tubes were centrifuged at 5,000 x g for 10 minutes and the supernatant discarded.

The pellets were resuspended with 180 µl gram-positive bacteria lysis buffer and incubated at 37 °C for 30 minutes. Then 200 µL of the lysis solution and 20 µL of Proteinase K, both included in the kit, were added to the tubes and the tubes were vortexed for 30 seconds each. The samples were then incubated at 56 °C for 35 minutes and vortexed about every ten minutes during the incubation until the cells of the lower concentration samples appeared to be completely lysed. After the addition of 20 µL of RNase A and vortexing, the samples were incubated at room temperature for 10 minutes.

According to the protocol instructions, 400 µL of 50% ethanol were to be added to each sample next. This was done for all samples, except S1T and S2T where 600 µL were added due to the high viscosity of these samples. The prepared lysates were then transferred to GeneJET Genomic DNA purification columns inserted into a collection tube. As a rule, all sample containing columns were centrifuged for 1 minute at 6,000 x g. Samples that still were in an inhomogeneous suspension after this step were centrifuged for 2 minutes at 12,000 x g until a uniform suspension was obtained. The collection tubes were then discarded and the columns placed in new, sterile collection tubes.

In the next step, 500 µL of washing buffer I was added to the columns which were then centrifuged for 1 minute at 8,000 x g. Tubes where the column still contained liquid were centrifuged for an additional 2 minutes at 11,500 x g. The

flow-through solution was discarded and the columns placed back into the collection tubes. After the addition of 500 µL of washing buffer II, the samples were centrifuged for 3 minutes at 13,000 x g, the collection tubes discarded and the purification columns placed into new collection tubes.

Finally, 200 µL of elution buffer was applied to the purification column membranes which were then incubated at room temperature for 2 minutes and centrifuged for 1 minute at 8,000 x g. Since collection tubes without lids were used to minimize the risk of parts detaching, the eluted samples were transferred to 1.5 mL Eppendorf tubes and stored at -20 °C.

PCR

PCR analysis for 16S rRNA was performed with forward primers 27F, 63F, 515F(Y), 519F and reverse primers 515R, 800Rmod, 806R, 926R, 1100R, 1387R. PCR analysis for housekeeping genes was performed with forward and reverse primer pairs for gyrB, DN13, S.myy, atpD, rpoD, efp and dnaK (Table 10).

Table 10. DNA sequences of primers used for PCR analysis.

Gene	Name	Primer	DNA sequence (5´-3´)
16S rRNA	16S Ribosome	27F*	AGAGTTTGATYMTGGCTCAG
16S rRNA	subunit	63F	CAGGCCTAACACATGCAAGTC
16S rRNA		515F(Y)	GTGYCAGCMGCCGCGGTAA
16S rRNA		519F	CAGCAGCCGCGGTAA
16S rRNA		515R	TTA CCG CGG CKG CTG GCA C
16S rRNA		800Rmod	GGACTACCAGGGTATCTAAT
16S rRNA		806R	GGACTACNVGGGTWTCTAAT
16S rRNA		926R	CCGYCAATTYMTTTRAGTTT
16S rRNA		1100R	AGGGTTGCGCTCGTTG
16S rRNA		1387R	CGGCGGWGTGTACAAGGC
gyrB	DNA gyrase,	gyrB.F	AGCATYAARGTGCTGAARGG
gyrB	subunit B	gyrB.R	GGTCATGATGATGATGTTGTG
DN13	Chaperone,	DN13.F(dnaJ)	AGCGGGATTATTATGAAC
DN13	Hsp40	DN13.R(dnaJ)	GACCGGTGTTTCTACAAC

S.myy	16S Ribosome	S.myy.F	GTGAGATGTTGGGTTAAG
S.myy	subunit	S.myy.R	GTCCCTACCATTGTAGTA
atpD	ATP synthase	atpD.R(Xa)	GGGCAAGATCGTTCAGAT
atpD		atpD.R(Xa)	GCTCTTGGTCGAGGTGAT
rpoD dnaK	Sigma factor	rpoD.F(Xa)	ATGGCCAACGAACGTCCTGC
rpoD dnaK		rpoD.R(Xa)	AACTTGTAACCGCGACGGTATTCG
Efp	Elongation	efp.F(Xa)	TCATCACCGAGACCGAATA
Efp	Factor	efp.R(Xa)	TCCTGGTTGACGAACAGC
dnaK	Chaperone	dnaF.R(Xa)	GGTATTGACCTCGGCACCAC
dnaK		dnaK.R(Xa)	ACCTTCGGCATACGGGTCT

**F=forward primer, R=reverse primer*

The combinations of the 16S rRNA primers yield a minimum sequence length of 291 bp (519F and 800Rmod) and a maximum sequence length of 1360 bp (27F and 1387R). In total these primers provide 22 different combinations. The sequence lengths yielded by the primer pairs for the housekeeping genes were read from the gel and were between 300 and 1500 bp.

According to the data sheet of the Taq Polymerase used (Thermo Scientific 2016b), PCR products of up to 2 kb in length can be produced within 1 minute which was suitable for all used sequences. The protocol recommends 5 µL 10X DreamTaq buffer, 5 µL dNTP Mix, between 0.1 and 1 µM of each forward and reverse primer, between 10 pg and 1 µg of template DNA, 1.25 U of DreamTaq DNA polymerase and then water for a 50 µL reaction. For this project 10 µL dNTP Mix, 2.5 µL of each forward and reverse primer and a volume of template DNA corresponding to 100 ng was used.

The amount of bacterial DNA in the tubes was 100 ng for ‘Small’ whereas the amount for ‘Large’ was chosen to be 50 ng due to the presumed size difference observed during dilution and the high viscosity observed during DNA purification. Hyper viscosity can be problematic in PCR as it slows down both denaturation of the sample DNA, annealing of the primers and extension of the primers.

Gel electrophoresis

Gel electrophoresis is a technique used to separate DNA molecules with respect to their size/length. A gel with small wells (where sample DNA is added) on one side is subjected to a current that pulls the negatively charged DNA fragments towards the positive electrode. Small fragments are pulled faster through the pores of the gel than large fragments that are retarded relative to their size. By using a reference sample containing DNA fragments of known sizes, the size of the sample DNA can be determined (Figure 25).

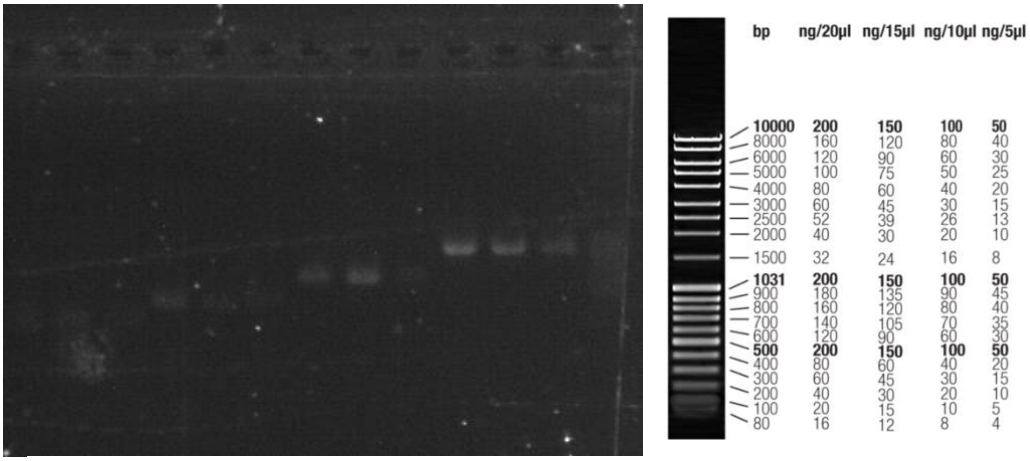

Figure 25. Gel electrophoresis result (left) and reference sample (right, adapted from Thermo Scientific 2016c)

The purpose of gel electrophoresis in this case was to verify that DNA was present in the samples after DNA-purification and that the lengths of these DNA-fragments roughly correspond to the expected length after PCR analysis. The expected length can be calculated from the known binding sites of the PCR primers used.

The gel was prepared by mixing 1.5% of agarose powder with 1x Tris-acetate-EDTA (TAE) buffer, heating up the mixture until the agarose powder was completely dissolved and adding 0.002% of Midori dye to make the DNA visible under UV light. Then the mixture was cooled down to around 60 °C before it was poured into a gel tray with combs to create the sample pockets. After the gel had solidified, the combs were removed and the gel submerged into 1xTAE buffer. The DNA samples to be examined were mixed with milliQ water and a loading dye in a 3:2:1 ratio. In addition, 6 µL of the pre-mixed ladder (Thermo Fisher 2016c) were added next to the pockets with the DNA samples. A current of around 100 V was applied to the gel for approximately 1 hour depending on how far the loading dye in the samples had migrated after 30 minutes. When the dye had migrated several

centimetres (but well before reaching the edge of the gel) the current was turned off, the gel photographed with the UV-light camera and stored at 4 °C for later use.

PCR Purification

Purification of the PCR products was accomplished using Thermo Scientific GeneJET PCR purification kit (Thermo Scientific 2015a). Since vacuum manifolds were not available, the protocol for purification with a centrifuge was followed. All centrifuging steps were carried out at 13,000 x g.

First, the PCR products were mixed with a binding buffer in a 1:1 ratio and the colour of the resulting solution verified to be yellow (for DNA-fragments smaller than 500 base-pairs, the solution was mixed with 100% isopropanol in a 2:1 ratio). The solution was then transferred to the GeneJET purification column, centrifuged for 1 minute and the flow-through discarded. Then 700 µL of wash buffer were added to the column, the column centrifuged for 1 minute and the flow-through discarded. To remove residual alcohol, the column was centrifuged for an additional 1 minute.

Finally, the GeneJET purification column was transferred to a clean 1.5 mL microcentrifuge tube, filled with 50 µL of elution buffer and centrifuged for 1 minute. The purification column was discarded and the microcentrifuge tubes stored at -20 °C.

Qubit™-measurement

The Qubit™ fluorometer was used to measure DNA quantity in a sample. Following the manual (Thermo Scientific 2015b) First, the working solution and two standards for calibration were prepared. The working solution consisted of the Qubit™ buffer and the Qubit™ reagent, mixed in a 200:1 ratio. The final volume in each Qubit™-tube was 200 µL. The tubes for the standards each contained 10 µL of the respective standard from the kit plus 190 µL of the working solution. The tubes for the samples contained 2 µL sample and 198 µL of working solution. After successful calibration with the two standards, the samples could be measured. The Qubit™ fluorometer could then calculate the amount of DNA in the sample if the sample volume was entered.

Sequencing

For sequencing, all DNA-samples were sent to Eurofins. Eurofins requires a sample volume of 10 µL consisting of 5 µL sample containing between 20 and 80 ng of DNA and 5 µL of primer with a concentration of 5mM. In some cases the amount of DNA in the samples had to be adapted through dilution or concentration of the sample, the primer or both in order to achieve optimal sequencing results. All samples were sent once with the forward and once with the reverse primers used in

PCR. Eurofins returned, among other data, both the part of the DNA-sequence deemed reliable by them, the complete DNA-sequence identified and the associated chromatogram which is a visual representation of a DNA-sample.

Analysis

Analysis of the sequences was performed with the online version of NCBI (National Center for Biotechnology Information) microbial nucleotide BLAST (Basic Local Alignment Search Tool) against databases with representative genomes only and optimized for highly similar sequences (Megablast) (NCBI 2019). Sequence alignment was performed with the online version of EMBL-EBI (European Molecular Biology Laboratory - European Bioinformatics Institute) Clustal Omega DNA (EMBL-EBI 2019) with default settings. Some sequences were analysed in-depth using chromatograms for correcting possibly faulty base identifications by Eurofins but the improvements for sequence identity and gap closure were too small and the schedule too tight to include this as a rule.

6.2.2 Biotests

All biotests except for the differential media test and the API test were performed according to lab instructions from course BI1248 (Pell & Passoth 2018).

Gram staining

This test is used to determine whether a bacterium is gram-positive or gram-negative. A drop of water was placed on a microscope slide, one bacterial colony was mixed with the water and the mixture was distributed evenly over a 2 x 2 cm area. After drying the sample, it was fixated by moving it through the flame of a Bunsen burner a couple of times. The specimen was stained with crystal violet, the excess dye rinsed off, and then treated with iodine solution. A decolourizer, consisting of 96% ethanol and water was used to wash off all excess dye. Finally, safranin was added for counterstaining, rinsed off and the specimen is dried. Gram-negative bacteria appeared red while gram-positive bacteria appeared violet.

Oxidase test

Cytochrome c is an enzyme bacterium use for respiration. Oxidases can oxidize cytochrome c which then reacts with the oxidase test reagent used for this test. Several bacterial colonies were transferred to an oxidase test strip and incubated at room temperature for a couple of seconds. A colour change of the test strip to dark blue within 20 seconds indicated a positive result. No colour change or a colour change after 20 seconds indicated a negative result.

Catalase test

This test determines whether a bacterium is able to produce the enzyme catalase which can decompose hydrogen peroxide to oxygen and water. Several bacterial colonies were transferred to a microscope slide and a drop of hydrogen peroxide was added. If a reaction occurred and bubbles were formed the bacterium was able to produce catalase.

Oxidation Fermentation test

Some bacteria can utilize glucose through oxidation or fermentation. Several bacterial colonies were transferred to two identical tubes with glucose broth. One of the tubes was sealed with paraffin-oil to create an anaerobe environment. Both tubes were then incubated at 28 °C for 24 hours. If the colour of the glucose broth in a tube had changed to yellow, the glucose in this tube had been utilized through fermentation. If the colour of the glucose broth was still violet and the broth clear, the bacterium had not utilized the glucose. If the glucose broth was violet and turbid, the glucose had been utilized by respiration.

Nitrate reduction test

This test determines whether a bacterium is able to reduce nitrate till nitrite or nitrate to ammonium via nitrite. One bacterial colony was transferred to a tube containing nitrate broth and incubated at 37 °C for 24 hours. At the end of the incubation period a nitrate test strip was submerged in the nitrate broth for one second. If both measurement zones were coloured either only nitrite or both nitrite and nitrate were present in the broth. If only the lower measurement zone was coloured only nitrate was present in the broth and if none of the measurement zones was coloured neither nitrate nor nitrite were present in the broth. The colour intensity of the measurement zone was an indication of the amount of nitrate/nitrite present in the broth and could be compared to a reference colour table.

Urease test

Certain bacteria are able to produce the enzyme urease which can hydrolyse urea, converting it to ammonia and carbon dioxide. Several bacterial colonies were transferred to a tube containing urea broth and incubated at 28 °C for 24 hours. If the urea broth was red at the end of the incubation period, the bacterium produced urease. If the urea broth was still yellow, the bacterium did not produce urease.

Antibiotic sensitivity test

Many microorganisms produce secondary metabolites, chemical substances which are not necessary for basic survival but are used to fight other microorganisms competing for resources. Secondary metabolites that are active against bacteria are

called antibiotics and specific resistance in bacteria can be used to help with identification. Several bacterial colonies were mixed with sterile NaCl-solution to a turbidity of McFarland 0.5. Then a sterile cotton swab was used to evenly distribute the mix on a 14 cm paper disc method (PDM) sensitivity medium plate. Wafers containing different antibiotics were placed on the plate and the plate incubated at 28 °C overnight. After the incubation period, the diameter of inhibition zones around the wafers could be measured and compared to a reference table to determine the bacterium's susceptibility or resistance to the specific antibiotics.

Differential media tests

These tests were performed to determine whether the bacteria could grow in the presence of 5% NaCl in the LBA mixture, on violet red bile agar, or on mannitol salt agar for comparison with literature.

API test

For the API 50 CHB/E test, a few bacterial colonies were suspended in 1 ml of sterile distilled water to a turbidity of McFarland 4. This suspension was transferred to an ampule of API 50 CHB/E medium included in the test kit and mixed to a homogenous solution. The solution was distributed to the 50 reagent wells and mineral oil was added to the wells for cover. The strips were incubated at 28 °C for 48 hours and colour shifts from the original red of the medium to orange or yellow were noted both after 24 and 48 hours. The original protocol for the API 50 CHB/E test is provided by Biomérieux (Biomérieux 2011).

6.3 Results & Discussion

6.3.1 Gene sequencing

The BLAST result webpage shows a list of the database hits matching the template sequence to a certain degree. The most important parts for the purpose of this thesis were 'Description' (name of the organism), 'Query Cover' (part of the template sequence overlapping the matched sequence in percent – a higher number equals a better match), 'E value' (the expected probability of a match occurring by chance – a lower value equals a better match) and 'Per. Ident' (part of the template sequence exactly matching the particular hits in percent – a higher number equals a better match) (Figure 26). The default sorting order is with increasing 'E value'.

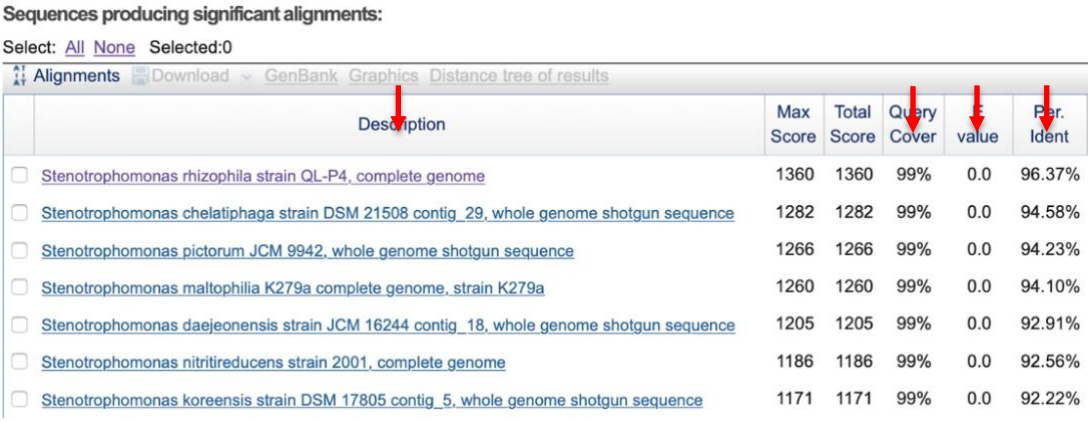

Figure 26. Example of a BLAST result from a 16S rRNA sequence.

A detailed result page, showing the positions of gaps and discrepancies in base identification is available through clicking on the organism name in 'Description' (Figure 27).

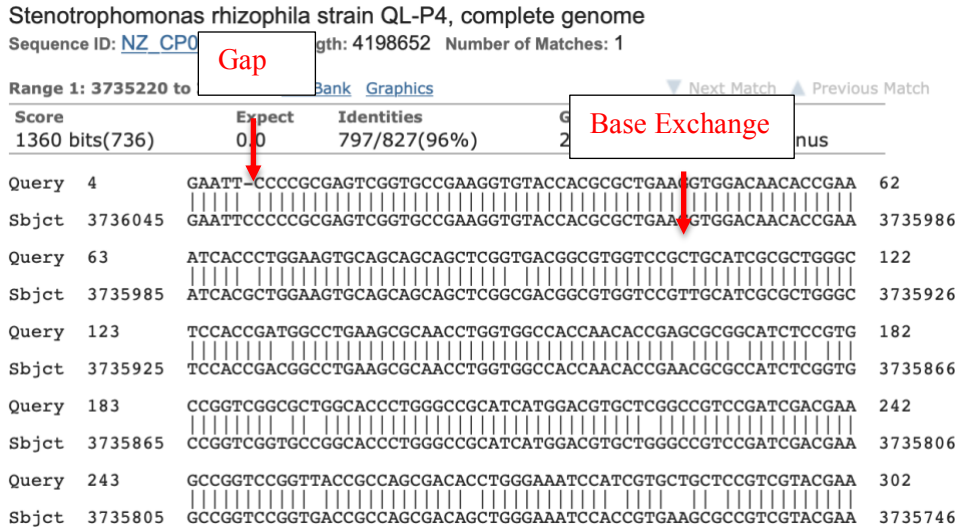

Figure 27. Detailed BLAST result page showing the positions of gaps and base identification discrepancies

The first BLAST analyses showed a variety of inconsequential hits, possibly due to the lack of practise on our side which resulted in short usable sequences with high E-values (Table 11).

Table 11. Early BLAST matches for bacteria 'Small' and 'Large' sorted by increasing E value

Primer	Sequence Length	Best Match	Query Cover	E value	Identity
	291	*Achromobacter xylosoxidans* genome assembly NCTC10807	100%	1.00E-141	98.28%
	112	*Candidatus Kinetoplastibacterium blastocrithidii* (ex *Angomonas deanei* ATCC 30255	100%	2.00E-49	99.11%
800Rmod	100	*Stenotrophomonas chelatiphaga* strain DSM 21508 contig_58	100%	4.00E-41	97.00%
519F	76	*Nevskia soli* DSM 19509 BP26DRAFT_scaffold00046.46_C	97%	5.00E-29	98.68%
800Rmod	86	*Stenotrophomonas rhizophila* strain QL-P4	90%	5.00E-28	95.35%
519F	63	*Azonexus hydrophilus* DSM 23864 G471DRAFT_scaffold00015.15_C	100%	2.00E-26	100.00%
800Rmod	65	*Pusillimonas noertemannii* BS8 Contig_48	81%	4.00E-25	98.46%
	46	*Lautropia mirabilis* ATCC 51599 SCAFFOLD3	100%	2.00E-13	97.83%
519F	8	-	-	-	-

Later sequencing results with longer usable sequences from 16S rRNA gene suggested that both bacteria were of the genus *Stenotrophomonas*. Both *S. maltophilia, S. rhizophila* continuously ranked among the top 5 hits (Table 12). Often, they were the top 2 hits where *S. maltophilia* more often ranked first.

Table 12. 16S rRNA BLAST matches for bacteria 'Small' and 'Large' sorted by increasing E value

Primer	Sequence Length	Best Match	Query Cover	E value	Per Ident
1100R	938	*Stenotrophomonas maltophilia* strain K279a	99%	0	99.04%
1387R	934	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.82%
1387R	986	*Stenotrophomonas maltophilia* strain K279a	99%	0	98.78%
1100R	1017	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.72%
1387R	1125	*Stenotrophomonas maltophilia* strain K279a	99%	0	98.67%
27F	919	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.59%
27F	999	*Stenotrophomonas maltophilia* strain K279a	99%	0	98.40%

27F	808	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.39%
27F	796	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.37%
1100R	1029	*Stenotrophomonas chelatiphaga* strain DSM 21508 contig_58	99%	0	98.35%
27F	774	*Stenotrophomonas chelatiphaga* strain DSM 21508 contig_58	99%	0	98.19%
27F	968	*Stenotrophomonas maltophilia* strain K279a	99%	0	98.04%
1100R	316	*Stenotrophomonas maltophilia* strain K279a	99%	8.00E-172	99.68%
27F	19	*Rhodococcus pyridinivorans* SB3094	100%	0.38	100.00%
1387R	17	*Lactobacillus parafarraginis* DSM 18390 = JCM 14109	89%	2.4	100.00%
27F	18	*Ruegeria marina* strain CGMCC 1.9108	51%	4.5	100.00%

Sequencing result of selected housekeeping genes yielded similar results but *S. rhizophila* was more often ranked first (Table 13). In addition, *Pseudomonas* and *Pseudoxanthomonas* ranked first a couple of times which could be a reason for *Stenotrophomonas* first being classified as *Xanthomonas* and *Pseudomonas* (Chang et al. 2015).

Table 13. Housekeeping gene BLAST matches for bacteria 'Small' and 'Large' sorted by E value

Primer	Sequence Length	Best Match	Query Cover	E value	Per Ident
dnaK.F	630	*Stenotrophomonas maltophilia* strain K279a	96%	0	97.46%
dnaK.F	968	*Stenotrophomonas maltophilia* strain K279a	98%	0	97.42%
dnaK.R	996	*Stenotrophomonas maltophilia* strain K279a	99%	0	97.39%
dnaK.R	759	*Stenotrophomonas maltophilia* strain K279a	98%	0	97.23%
atpD.F	816	Stenotrophomonas rhizophila strain QL-P4	97%	0	96.57%
atpD.R	804	*Stenotrophomonas rhizophila* strain QL-P4	97%	0	96.39%
atpD.R	819	*Stenotrophomonas rhizophila* strain QL-P4	99%	0	96.34%

atpD.F	814	*Stenotrophomonas rhizophila* strain QL-P4	98%	0	96.31%
gyrB.F	1280	*Pseudomonas fluorescens* F113	99%	0	92.50%
gyrB.R	1181	*Pseudomonas fluorescens* F113	98%	0	92.21%
rpoD.F	1060	*Stenotrophomonas maltophilia* strain K279a	98%	0	90.47%
rpoD.F	1140	*Stenotrophomonas maltophilia* strain K279a	99%	0	90.44%
rpoD.R	1092	*Stenotrophomonas maltophilia* strain K279a	99%	0	90.02%
rpoD.R	1024	*Stenotrophomonas maltophilia* strain K279a	98%	0	89.94%
efp.F	394	*Pseudomonas fuscovaginae* strain LMG 2158 genome assembly	44%	8.00E-73	79.70%
S.myy.F	138	*Stenotrophomonas chelatiphaga* strain DSM 21508 contig_58	97%	9.00E-71	100.00%
S.myy.R	130	*Pseudoxanthomonas suwonensis* J47 Psesu4DRAFT_scaffold_41.42_C	99%	5.00E-66	100.00%
S.myy.R	129	*Pseudoxanthomonas suwonensis* J47 Psesu4DRAFT_scaffold_41.42_C	100%	2.00E-65	100.00%
S.myy.F	122	*Lysobacter tolerans* strain UM1	97%	2.00E-56	99.18%
efp.R	18	*Arthrobacter globiformis* NBRC 12137	100%	6.00E-01	100.00%

These results indicated that both bacteria belong to the genus *Stenotrophomonas*. Chun et al. (2018) suggest that a 16S rRNA sequence similarity less than 98.7% between the best match and the type strain in the database indicates that a DNA sample represents a new species. However, a sequence similarity of at least 98.7% does not necessarily mean that the DNA sample belongs to the type strain species according to the authors. Factors that should be taken into account from an analysis perspective are the overall genome related index (OGRI) and phylogenomic treeing. OGRI is a measure of similarity between two genome sequences and can be measured in average nucleotide identity (ANI) or digital DNA-DNA hybridization (DDH). Identification as a known species is only valid if ANI is above 95% or dDDH is above 70% (Figure 28).

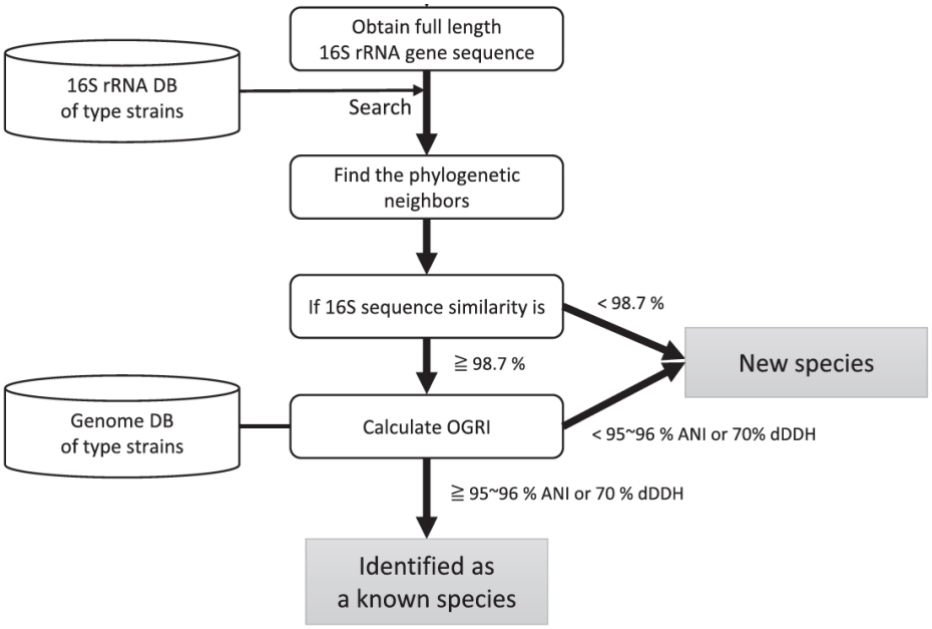

Figure 28. Classification workflow with standards for sequence similarity and genome relatedness (adapted from Chun et al. 2018)

In this project ANI and dDDH were not calculated but the best match with 16S rRNA was *S. maltophilia* (Table 14), closely followed by *S. rhizophila*.

Table 14. 16S rRNA BLAST matches for bacteria 'Small' and 'Large' with over 98.7% identity and E value

Primer	Sequence Length	Best Match	Query Cover	E value	Per Ident
1100R	938	*Stenotrophomonas maltophilia* strain K279a	99%	0	99.04%
1387R	934	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.82%
1387R	986	*Stenotrophomonas maltophilia* strain K279a	99%	0	98.78%
1100R	1017	*Stenotrophomonas maltophilia* strain K279a	100%	0	98.72%

S. maltophilia is an opportunistic human pathogen with a multidrug resistant profile (Chang et al. 2015). We therefore considered the risks, and decided not to pursue further work to confirm the identity as *S. maltophilia*.

6.3.2 Biotests

Gram staining revealed that both bacteria were gram-negative which is in line with *S. maltophilia*.

The catalase and oxidase tests were both negative for bacterium 'Small' and both positive for bacterium 'Large'. One article describes certain strains of *S. maltophilia* as catalase-positive and usually oxidase-positive (Adegoke et al. 2017). Heylen et. al (2017) characterized a certain strain of *S. rhizophila* as oxidase-positive while two other strains of *S. maltophilia* were oxidase/negative. The article also described one of the tested *S. maltophilia* strains as nitrate reduction negative and the other one, as well as *S. rhizophila*, as nitrate reduction positive. Our bacteria were both able to reduce nitrate. The authors also introduce *S. terrae* and *S. humi* which are in some cases very similar to, and in some cases very different from, *S. maltophilia* and *S. rhizophila*. The oxidation fermentation (OF) test and the urease test were both negative for both bacteria but we could not find any references in literature for these test (Table 15).

Table 15. Biotest results for 'Small'(1), 'Large' (2), S. maltophilia LMG 958 (3), S. maltophilia LMG 22072 (4) and S. rhizophila CCUG 47042 (5). Results for (3), (4) and (5) from Heylen et. al (2017)

Test name	1	2	3	4	5
Catalase	-	-	+*	+*	n/a
Oxidase	-	-	-	-	+
Nitrate reduction	+	+	+	-	+
OF	-	-	n/a	n/a	n/a
Urease	-	-	n/a	n/a	n/a

*Data from Adegoke et al. (2017). No specific strains mentioned for this data.

Both bacteria were somewhat sensitive to Penicillin G, Sulphamethoxazole, Chloramphenicol and Sulphamethoxazole/Trimethoprim but resistant to Erythromycin, which did not fit the multidrug resistant profile of *S. maltophilia* (Table 16).

Table 16. Resistance of bacteria 'Small' and 'Large' to antibiotics

Antibiotic name	Bacterium 'Small'	Bacterium 'Large'
Penicillin G	sensitive	sensitive
Sulphamethoxazole	sensitive	sensitive
Chloramphenicol	sensitive	sensitive
Sulphamethoxazole/Trimethoprim	sensitive	sensitive
Erythromycin	resistant	resistant

Both bacteria could grow on violet red bile agar but not on mannitol salt agar, but we did not find any direct references in literature for comparison. Both bacteria could somewhat grow on LBA with 5% NaCl but in literature (Wolf et al. 2002) we found indications that *S. maltophilia* had 0% growth on 5% NaCl-LBA while *S. rhizophila* had 100% growth on 5% NaCl-LBA which placed our bacteria somewhere in between.

The API 50 test result (Table 17) did not match any of the results found for *S. maltophilia* or *S. rhizophila* we found in literature, but we were not sure whether a mismatch would in turn rule out the bacteria's' identity being *S. maltophilia*, *rhizophila* or some other strain.

Table 17. API 50 test results for 'Small'(1), 'Large' (2), S. maltophilia LMG 958 (3), S. maltophilia LMG 22072 (4) and S. rhizophila CCUG 47042 (5). Results for (3), (4) and (5) from Heylen et. al (2017) and are noted as positive or negative. Only substrates with positive reactions are listed.

Substrate name	1	2	3	4	5
Glycerol	-	+++	-	-	-
L-Arabinose	-	++++	-	-	-
D-Xylose	-	+++	-	-	-
D-Galactose	-	++++	-	-	-
D-Glucose	++	++++	+	+	+
D-Fructose	-	++	+	+	+
D-Mannose	+	++++	+	+	+
D-Mannitol	+	++++	-	-	-
Esculin, ferric citrate	brown	red/brown	+	+	+
D-Melibiose	-	++	-	+	-
D-Saccharose	-	+++	+++	+++	+++
Gentiobiose	-	+	+	+	-
D-Fucose	-	+	-	-	-

In summary, our biotests and the literature analyses of different types and strains of *Stenotrophomonas* lead to the conclusion that we could neither definitively confirm nor rule out the bacteria's identities. Different strains can have very different or very similar results and the exact test methods are not always specified.

6.3.3 *Stenotrophomonas*

One general problem with the study and identification of bacteria is that criteria for bacterial systematics and naming systems are changing over time. Earlier approaches for classification were mainly based on phenotypic and biochemical properties. As mentioned before, these properties can both differ between strains of the same bacteria and coincide with completely different bacteria. Identification based on DNA sequencing has in many cases provided new insights into the relationships between bacteria and has in itself evolved over time, both regarding sequencing accuracy, reliability and as a consequence the criteria for sequence identity. More recently, whole genome sequencing of bacteria has become common, enabling more robust and accurate analysis. Identification results using different methods or using older and newer variants of the same methods may therefore not be the same. This development has in many cases led to re-classification and re-naming of bacteria which can make it difficult to use and compare current results with data in older literature because of uncertainties of their true nature. However, a serious attempt to identify the isolated bacteria was made, leading to the following conclusions.

BLAST analysis of the DNA sequences of the second PCR analysis indicated that the bacteria might belong to *Stenotrophomonas*. The number one hit (97.5% sequence identity) obtained was *S. maltophilia* closely followed by *S. rhizophila*. Although far from certain, being based on partial gene sequencing and phenotyping, this result was regarded as a qualified guess of what bacteria it might be.

The genus *Stenotrophomonas* presently includes 12 species. These bacteria are described as abundant in nature (Klingenberg 2011) and seems to play an important role in cycling of elements such as nitrogen and sulphur (Ryan et al. 2009) (Ikemoto et al. 1980).

S. pavanii, *S. rhizophilia* and *S. maltophilia* are species known to associate with plants in a non-pathogenic interaction (Klingenberg, 2011). Instead they seem to have beneficial interactions with plants and therefore might be promising for applications in agriculture (Ryan et al, 2009).

Stenotrophomonas strains associated with plants are described to be endophytic. In contrast to epiphytic PGPRs, the mechanisms regarding growth promotion by endophytes are not as well known. Though most of the endophytic bacteria are facultative endophytes, one would assume that they share many of the properties that epiphytes possess (Hallmann et al. 1997, Berg & Hallman 2006). Just as in the case with epiphytic PGPRs, the mechanisms behind growth promoting effects can vary and often is a combination of several factors (Rosenblueth & Martínez-Romero 2006).

Different strains of *S. maltophilia*, which appears to be the most abundant, have been found to have growth promoting effects as well as being able of controlling plant fungal pathogens (Klingenberg 2011). The growth promoting effect of different *Stenotrophomonas* strains seems to be most prominent under highly saline conditions. It was reported that the yield of tomato, pepper, melon, carrot and wheat increased up to 180% after inoculation with *Stenotrophomonas* during trials in highly salinized soils (Ryan et al. 2009).

In a project done by (Klingenberg 2011) several strains of *Stenotrophomonas* were isolated from *Calliandra* plants. Of these strains, 31 showed some growth promoting abilities in vitro. The strains did not seem to have the ability to solubilize phosphorous or produce siderophores or cellulases but tested positive to produce lipases, proteases and indole acetic acid (IAA) (Klingenberg 2011). However, other studies reported the ability to solubilize phosphate (De Freitas et al. 1997) and produce siderophores (Minkwitz & Berg 2001). The production of lipases and proteases is regarded as antagonistic against phytopathogens while the production of a phytohormone like IAA directly promotes growth of the plant. General growth promotion was significant as legumes were inoculated with a combination of *Stenotrophomonas* strains isolated from the *Callandria* plant combined with different strains of rhizobium (Klingenberg 2011).

There are several other antagonistic substances produced by *Stenotrohophomonas spp* that have been identified. Among them are xantobaccin, oligomycin A, zwittermicin A and kanosamin. The production of these antibiotics *in vitro*, is environmentally variable and depends on factors such as pH, temperature and accessibility of minerals and nutrients (Compant et al, 2005, Lugtenberg & Kamilova, 2009). Despite the production of these substances and results that show antagonism against fungal pathogens *in vitro*, the knowledge of the actual significance of these processes inside the root tissue is very poor (Berg & Hallmann, 2006).

A dilemma though, seems to be that strains of *S. maltophilia* also can be human opportunistic pathogens, especially in immunosuppressed patients, with multidrug resistance towards antibiotics (Klingenberg, 2011). Hence such strains may not be appropriate to develop for commercial applications in agriculture (Binks et al, 1995). Other species from environmental isolates on the other hand, such as *S. rhizophilia*, does not share the human pathogenic traits. Although closely related, *S. rhizophilia* has a lower temperature optimum and does not prefer to grow at the human body temperature of 37°C, and should therefore be regarded as safe to use (Minkwitz and Berg, 2001), (Wolf et al, 2002). Ribbeck-Busch et al, 2005; Hagemann et al, 2006).

Even though we did not obtain any results that indicated growth promoting effects by the isolated bacteria in our experiment, 16S rDNA and housekeeping gene sequencing led us to believe that our bacteria belong to the genus

Stenotrophomonas. A genus where some strains have previously demonstrated growth promotion abilities for other crops. The growth promotive effects are as earlier mentioned most prominent during highly salinized conditions, where the protective mechanisms towards soil borne pathogens are mostly indirect.

The fact that *Stenotrophomonas* strains can be beneficial to plants at least gave us confidence that our strategy to screen for novel plant associated bacteria is valid. Despite our results not being significant, we make the conclusion that our bacterial isolates should not be disregarded. With the reference of previously published experiments, strains within the genus *Stenotrophomonas* could have a positive impact on plant growth under certain conditions. Therefore, further tests could be a subject of study for our isolates.

Despite the fact that no solid evidence for the ability of our strains to colonize potato plants were obtained, one also must take into account that the bacterial ability to colonize tubers and sprouts is likely to depend on the potato variety. So, a more precise execution of the experiments, would be to use the same potato variety as we used during the screening process. The reason for changing the variety was because of generally bad quality of the present material, probably because of too long storage.

7 Conclusion and future challenges

7.1 Conclusion

In summary we would like to thank our supervisors for having the patience to guide us through this process. We would like to mention the fact that we have learned a lot and achieved a lot.

- Two "new" bacteria were isolated
- The PGPR effect on potatoes was missing or weak
- The biopesticide effect could not be evaluated
- Indication that the bacteria belong to Stenotrophomonas.
- Bioassays and DNA analysis showed no exact match with known bacterial strains
- We have experienced how challenging it is to try to identify and develop a PGPR / biopesticide.

7.2 Future challenges

A future task could try and isolate additional plant associated and beneficial bacteria, but also to study and try to understand the key factors that are involved in the colonization process between PGPRs and plants. It is crucial that the establishment between plants and PGPRs works under field conditions in order to

obtain the effects, that for example a seed (or vegetative part) coating treatment with bacteria might have. Progress in this area need studies of the mechanisms that are involved in the process, both from the bacteria but also from the plant (and different varieties).

A recurrent problem with commercialized bacterial biopesticides seems to be fluctuating results causing dissatisfaction from the users. Even when independent product tests have been performed there are varying results where the previously demonstrated effects (by the producers) are either low or absent.

A challenge for the future is to put further research and development into this subject, is to understand the underlying factors for varying effects of plant-associated bacteria on crop performance under field conditions. It is possible that it could be because of practical errors in the production line (i.e. contamination of bacterial cultures or spontaneous mutations changing bacterial properties over time) resulting in batches with improper quality.

Based on the literature, a probable reason for failure during field conditions could be insufficient rhizosphere competence (ability to colonize the rhizosphere under different field conditions and maintain population density over time). The process of colonization is critical in order for the bacterial biopesticide to exert its effect, something that does not have to be taken into consideration with chemical or physical pesticide treatments. The effect of the bacterial application can vary depending on structure and chemistry of the soil, but also on the biological composition of the soil. Namely, if the microbial activity of the present soil flora is high, one could assume a lot of competition towards the applied bacterial treatment. This hypothesis is substantiated by the fact that the effect of root colonizing bacteria often is most apparent during gnotobiotic circumstances compared to the effect in non-sterile soil.

To improve the colonization of beneficial root colonizing rhizobacteria researchers at SLU use nanoparticles to improve adhesion to roots. In one study the bacteria (*Bacillus amyloliquefaciens* UCMB 5113) clustered around TiO_2 nanoparticles increased adhesion on the root of oilseed rape and protection to fungal pathogens (Kessler et al, 2015). So, this might be a strategy to create a more favorable environment for root associated bacteria.

Another plausible cause to insufficient colonization has to do with the plant-microbe interaction. As previously mentioned, the colonization process within the rhizosphere is not a one-way communication process. It is not just the bacteria that has to be evaluated, but likewise the ability of the plant to become colonized. Further, the ability of a plant to be colonized vary to a large extent not just between different crops, but also between varieties within the same crop. This is something we have observed in person as we assisted experiments with *B. amyloliquefaciens* UCMB 5113 and inoculated seeds of rapeseed varieties and studied the effects. A current subject of study by Johan Meijer is to study variation in the ability of *Bacillus* 5113 to colonize different varieties of oilseed rape depending on the root exudate composition. It is important to understand, which varieties within a certain crop are compatible with the bacteria one would like to apply. But it might also be

of interest to crop breeding programs to breed for new crop varieties compatible with certain strains of bacteria that show growth promotive qualities.

Fluctuating results with biological biocontrol agents might be a constantly recurring problem. Living microorganisms that are to be applied seem sensitive to a variable environment combined with the fact that the plant-microbe interaction is built on a complex interplay that might make them vulnerable as a robust solution to pest control solely on its own. Hence, the incitement to use chemical pesticides with a more reliable effect independent of such circumstances. Maybe research regarding the plant-microbe interaction, the process of colonization and the understanding of what can be done to create a more hospitable environment around the rhizosphere, specifically for PGPRs, will be of greater importance in the future. As mentioned, plenty of bacterial beneficial effects to plants have been identified during *in vitro* trials, although the success rate under field conditions is far less. Tools to improve efficacy for such technology transfer deserve further studies.

The overall advantages with application of PGPRs (specifically biocontrol agents) is the multifaceted effects obtained by one application. As previously mentioned, a PGPR could work as both a biopesticide, a biostimulator and a biofertilizer. The theoretical benefit of a biopesticide in comparison to a chemical could be both environmental, financial as well as durable (reduced risk of pest resistance development). Environmentally one would assume a reduction of the chemical use, as the chemicals produced by the bacterial biopesticides (antibiosis) is localized to the root surface. As the mechanisms of biological control also would be based on competition and ISR, one would also assume a reduced presence of chemicals, which also would lead to a reduced risk of resistance development (multifaceted mechanism of action). Since bacteria is a living organism one might also bring up the concept of coevolution, where the biocontrol agent is adaptable and changes its genome along with changes in the pathogen. Although this implies that one would have to isolate the applied bacteria occasionally, in order to harvest bacteria under the process of natural selection. Economically it would be a benefit if seeds with bacterial coating were used and provided a persistent effect throughout the growth season, somewhat like a chemical pesticide that operates systemically. This assumes that the beneficial bacteria can withstand critical population density in the rhizosphere during a longer period.

References

Adegoke, A. A., Stenström, T. A., Okoh, A. I. (2017). *Stenotrophomonas maltophilia* as an Emerging Ubiquitous Pathogen: Looking Beyond Contemporary Antibiotic Therapy. Frontiers in Microbiology 8, 2276. DOI: 10.3389/fmicb.2017.02276

Antoun H, Kloepper JW (2001). *Plant growth promoting rhizobacteria* . In: Brenner S, Miller JH, editors. Encyclopedia of Genetics. Academic; New York. pp. 1477–1480. [Google Scholar]

Bardgett RD, van der Putten WH (2014). *Belowground biodiversity and ecosystem functioning*. Nature 515:505-511. DOI: 10.1038/nature13855

Binks, P.R., Nicklin, S., Bruce, N.C. (1995) *Degradation of RDX by Stenotrophomonas maltophilia PB1.* Applied and Environmental Microbiology 61: 1318–1322.

Biomérieux (2011). Product Information – api® 50 CHB/E Medium REF 50430, 07964G – en – 2011/07. Available: https://www.mediray.co.nz/media/15798/om_biomerieux_test-kits_ot-50430_package_insert-50430.pdf [2019-06-25]

Bloemberg G., Lugtenberg B (2001). *Molecular basis of plant growth promotion and biocontrol by rhizobacteria*. Current Opinion in Plant Biology 4:343-350

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E., Schulze-Lefert, P. (2013). *Structure and functions of the bacterial microbiota of plants.* Annual Review Plant Biology 64:807-838. doi: 10.1146/annurev-arplant-050312-120106

Chang, Y. T., Lin, C. Y., Chen, Y. H., Hsueh, P. (2015) Update on infections caused by *Stenotrophomonas maltophilia* with particular attention to resistance mechanisms and therapeutic options. Frontiers in Microbiology Sep 2;6:893. DOI:10.3389/fmicb.2015.00893

Chun, J., Oren, A., Ventosa, A., Christensen, H., Arahal, D. R., da Costa, M. S., Rooney, A. P., Yi, H., Xu, X., De Meyer, S., Trujillo, M. E. (2018) Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. International Journal of Systematic and Evolutionary Microbiology 68: 461-466. DOI: 10.1099/ijsem.0.002516

Compant, S., Clément, C. and Sessitsch, A. (2010) *Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization*. Soil Biology and Biochemistry 42: 669-678.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A. (2005) *Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospect*s. Applied and Environmental Microbiology 71: 4951-4959

De Freitas, J.R., Banerjee, M.R., Germida, J.J. (1997) *Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus intake of canola (Brassica napus)*. Biology and Fertility of Soils 24: 358-364

Dixelius, C. (2012) *Phytophthora växtförstöraren [Broschyr].* Swedish university of agricultural sciences (SLU). https://bioresurs.uu.se/gmo/pdf/Phytophthora_vaxtforstoraren.pdf [2019-05-14]

dos Santos, M.L., Berlitz, D.L., Wiest, S.L.F., Schünemann, R., Knaak, N., Fiuza, L.M. (2018). Benefits associated with the interaction of endophytic bacteria and plants. Brazilian Archives of Biology and Technology Vol.61: e1816043. http://dx.doi.org/10.1590/1678-4324-2018160431 ISSN 1678-4324 Online Edition. [2019-05-14]

European Molecular Biology Laboratory - European Bioinformatics Institute (2019). *Clustal Omega – Multiple Sequence Alignment.* Available: http://www.ebi.ac.uk/Tools/msa/clustalo/ [2019-06-25]

Gkarmiri, K. (2018) *Interactions of fungal pathogens and antagonistic bacteria in the rhizosphere of Brassica napus.* Acta Universitatis agriculturae Sueciae, 1652-6880 ; 2018:4 ISBN 978-91-7760-148-7.

Glick, B.R. (1995) *The enhancement of plant growth by free-living bacteria.* Canadian Journal of Microbiology 41:109–117.

Gray EJ, Smith DL. (2005) *Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signalling processes*. Soil Biology and Biochemistry 37:395–412.

Grönberg, L., Anderson, B., Yuen, J. (2011) Can Weed Hosts Increase Aggressiveness of *Phytophthora infestans* on Potato? Phytopathology 102: 429-433. DOI: 10.1094/PHYTO-07-11-0192

Hagemann, M., Hasse, D., Berg, G. (2006) Detection of a phage genome carrying a zonula occludens like toxin gene (zot) in clinical isolates of Stenotrophomonas maltophilia. Archives of Microbiology 185:449–458

Hallmann, J., Berg, G. (2006) Spectrum and population dynamics of bacterial root endophytes. In: Schulz, B., Boyle, C. och Sieber, T.N. (eds.). Microbial Root Endophytes. Soil Biology, Vol 9. Springer-Verlag Berlin Heidelberg. pp. 15-31.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. och Kloepper, J.W. (1997) Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43: 895-914.

Hardoim, P.R., van Overbeek, L.S. och van Elsas, J.D. (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology 16: 463- 471.

Hammerschmidt R, (1999) Induced disease resistance: how to induced plants stop pathogens ? Physiological and Molecular Plant Pathology 55: 77-84

Hentschel et al., U. Hentschel, M. Steinert, J. Hacker (2000) Common molecular mechanisms of symbiosis and pathogenesis.Trends in Microbiology 8: 226-231

Heylen, K., Vanparys, B., Peirsegaele, F., Lebbe, L., De Vos, P. (2007) *Stenotrophomonas terrae* sp. nov. and *Stenotrophomonas humi* sp. nov., two nitrate-reducing bacteria isolated from soil. International Journal of Systematic Evolutionary Microbiology 57:2056-2061. DOI:10.1099/ijs.0.65044-0

Hiltner L (1904) Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Gründüngung und Brache. Arbeiten der Deutschen Landwirtschaftlichen Gesellschaft 98: 59–78.(About new experiences and problems in the field of Bodenbakteriologie)

Hirsch, A. M. (2004) *Plant-microbe symbioses: a continuum from commensalism to parasitism.* Symbiosis 37: 345-363.

Ikemoto, S., Suzuki, K., Kaneko, T. and Komagata, K. (1980) *Characterization of strains of Pseudomonas maltophilia which do not require methionine*. International Journal of Systematic Bacteriology 30: 437–447.

Janda, J. M. & Abbot, S. L. (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal of Clinical Microbiology 45: 2761-2764. DOI: 10.1128/JCM.01228-07

Jones, D.L., Nguyen, C., Finlay, R.D. (2009). *Carbon flow in the rhizosphere: carbon trading at the soil-root interface.* Plant and Soil 321: 5-33.

Judelson, H. S., Blanco, F. A. (2005). *The spores of Phytophthora: weapons of the plant destroyer*. Nature Reviews Microbiology 3: 47–58.

Kamal, R., Gusain, Y.S., Kumar,V., Sharma, A.K. (2015) *Disease management through biological control agents: An eco-friendly and cost effective approach for sustainable agriculture-* A Review. Agricultural Review, 36: 37-45. [https://www.arccjournals.com/uploads/articles/4R1464.pdf]

Klingenberg, D. (2011). *Diversitet och tillväxtfrämjande egenskaper hos Stenotrophomonas-endofyter isolerade från Calliandra calothyrsus meisn. Rotknölar.* Magistersavhandling Institutionen för livsmedels- och miljövetenskaper Mikrobiologi. Helsingfors Universitet.

Kloepper JW, Lifshitz R, Zablotowicz RM. (1989) *Free-living bacterial inocula for enhancing crop productity*. Trends in Biotechnology 7:39–43. [Google Scholar]

Lugtenberg, B., Kamilova, F. (2009) *Plant-growth-promoting rhizobacteria*. Annual Review of Microbiology 63: 541-556.

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A. (2008) Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus Ensifer (including former Sinorhizobium). International Journal of Systematic and Evolutionary Microbiology (2008), 58, 200–214. DOI:10.1099/ijs.0.65392-0

Minkwitz, A. och Berg, G. (2001) *Comparison of antifungal activities and 16S ribosomal DNA sequences of clinical and environmental isolates of Stenotrophomonas maltophilia*. Journal of Clinical Microbiology 39: 139-145.

National Center for Biotechnology Information (2019). *Basic Local Alignment Search Tool.* Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi [2019-06-25]

Palmqvist, N.G., Bejai, S., Meijer, J., Seisenbaeva, G.A., Kessler, V.G. (2015) Nano titania aided clustering and adhesion of beneficial bacteria to plant roots to enhance crop growth and stress management. Scientific Reports May 13;5:10146. doi: 10.1038/srep10146.

Pankhurst, C.E., Lynch, J.M. (2005). *Biological control of soil-borne plant disease.* In: Encyclopedia of Soils in the Environment. Vol.1. (ed D. Hillel) Elsevier, Oxford, pp.129-135. [https://www.sciencedirect.com/topics/earth-and-planetary-sciences/biocontrol-agent]

Pell, M., Passoth,V. (2018) Kurskompendium BI1248, VT 2018. Institutionen för mikrobiologi, SLU (April 2008)

Philippot, L., Raaijmakers, J.M., Lemanceau, P., van der Putten, W.H. (2013). *Going back to the roots: the microbial ecology of the rhizosphere.* Nature Reviews Microbiology, 11: 789-799.

Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., van Loon, L.C. (1998). *A novel signaling pathway controlling induced systemic resistance in Arabidopsis*. Plant Cell 10:1571–1580

Pieterse, C. M. J., Van Wees, S. C. M., Ton, J., Van Pelt, J. A., Van Loon, L. C. (2002). *Signalling in rhizobacteria induced systemic resistance in Arabidopsis thaliana*. Plant Biology 4:535-544

Podile AR, Kishore GK. (2006) *Plant growth-promoting rhizobacteria*. In: Gnanamanickam SS, editor. Plant-Associated Bacteria. Springer; Netherlands: pp. 195–230.

Ribbeck-Busch K, Roder A, Hasse D, de Boer W, Martínez JL, Hagemann M, Berg G (2005) *A molecular biological protocol to distinguish potentially human pathogenic Stenotrophomonas maltophilia from plant-associated Stenotrophomonas rhizophila*. Environmental Microbiology 7:1853–1858

Rosenblueth, M., Martínez-Romero, E. (2006) *Bacterial endophytes and their interactions with hosts*. Molecular Plant-Microbe Interactions 19: 827-837.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., ven der Lelie, D., Dow, J.M. (2009) *The versatility and adaptation of bacteria from the genus Stenotrophomonas*. Nature Reviews Microbiology 7: 514-525

SCB, *Växtskyddsmedel i jord- och trädgårdsbruket (*2006) 18 december, Användning i grödor. Available: https://www.scb.se/hitta-statistik/statistik-efter-amne/miljo/kemikalier-forsaljning-och-anvandning/vaxtskyddsmedel-i-jord--och-tradgardsbruket--anvandning-i-grodor/ [2019-05-14]

Singh, J.S. (2013). *Plant Growth Promoting Rhizobacteria Potential Microbes for Sustainable Agriculture.* Resonance 18: 275-281. *Available:* https://link.springer.com/article/10.1007/s12045-013-0038-y [2019-05-14]

Thermo Scientific (2015a). Product Information – *Thermo Scientific GeneJET PCR Purification Kit #K0701, #K0702. 2015 (Rev. 10)*. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012662_GeneJET_PCR_Purification_UG.pdf [2019-06-25]

Thermo Scientific (2015b). Product Information - *Qubit® dsDNA HS Assay Kits. Catalog nos. Q32851, Q32854*. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf [2019-06-25]

Thermo Scientific (2016a). Product Information – *Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721, #K0722. Pub. No. MAN0012663, Rev. Date 12 October 2016 (Rev. B.00)*. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012663_GeneJET_Genomic_DNA_Purification_Kit_UG.pdf [2019-06-25]

Thermo Scientific (2016b). Product Information – *Thermo Scientific DreamTaq DNA Polymerase. Pub. No. MAN0012036, Rev. Date 23 December 2016 (Rev. B.00)*. Available: https://assets.fishersci.com/TFS-Assets/LSG/manuals/MAN0012036_DreamTaqDNAPolymerase_500U_UG.pdf [2019-06-25]

Thermo Scientific (2016c). Product Information – *Thermo Scientific MassRuler DNA Ladder Mix. Pub. No. MAN0013021, Rev. Date 9 December 2016 (Rev. B.00)*. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013021_MassRuler_DNALadder_RTU_UG.pdf [2019-06-25]

Wang, Z-Y. (2017) *Enhancing Tropane Alkaloid Production Based on the Functional Identification of Tropine-Forming Reductase in Scopolia lurida, a Tibetan Medicinal Plant) Zeng-Yu Wang 2017.* Front Plant Sci. 2017; 8: 1745. Published online 2017 Oct 16. doi: 10.3389/fpls.2017.01745

van Wees, SCM., E.A.M. de Swart, J.A. van Pelt, L.C. van Loon, and C.M.J. Pieterse. (2000). *Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-bacterial spot in bell pepper induced by acibenzolar-S-methyl. Plant dependent defense pathways in Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA 97: 8711–8716

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Biology 52: 487-511.

Woese, C.R., Fox, G.E. (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proceedings of the National Academy of Sciences USA 74: 5088-5090. DOI: 10.1073/pnas.74.11.5088

Wolf A, Fritze A, Hagemann M, Berg G (2002) *Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties*. International Journal of Systematic and Evolutionary Microbiology 52:1937–1944

Yang, B., Wang, Y., Qian, P. (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics 17:135. DOI: 10.1186/s12859-016-0992-y

Zhao, K., Zeng, J., Zhao, T., Zhang, H., Qiu, F., Yang, C., Zeng, L., Liu, X., Chen, M., Lan, X., Liao, Z. (2017).*Enhancing tropane alkaloid production based on the functional identification of tropine-forming reductase in Scopolia lurida, a Tibetan medicinal plant.* Frontiers in Plant Science Oct 16;8:1745. doi: 10.3389/fpls.2017.01745. eCollection 2017

Acknowledgements

Thanks to our supervisor Johan Meijer as well as our co-supervisors Björn Andersson and Mikael Pell for putting in a lot of effort to guide us through this work.

Nr 171

Uppsala 2020

Department of Plant Biology SLU

Box 7080

75007 Uppsala, Sweden